Short Communication

Mutations in the NS1 C-terminal tail do not enhance replication or virulence of the 2009 pandemic H1N1 influenza A virus

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The 'classical' swine H1N1 influenza A virus lineage was established after the devastating 1918 human pandemic virus entered domestic pig herds. A descendent of this lineage recently re-emerged in humans as the 2009 pandemic H1N1 virus. Adaptation in pigs has led to several changes in the multifunctional viral NS1 protein as compared with the parental 1918 virus, most notably a K217E substitution that abolishes binding to host Crk/CrkL signalling adapters, and an 11 aa C-terminal truncation. Using reverse genetics, we reintroduced both these features into a prototype 2009 H1N1 strain, A/California/04/09. Restoration of Crk/CrkL binding or extension of NS1 to 230 aa had no impact on virus replication in human or swine cells. In addition, minimal effects on replication, pathogenicity and transmission were observed in mouse and ferret models. Our data suggest that the currently circulating 2009 H1N1 virus is optimized to replicate efficiently without requiring certain NS1 functions.

Since emerging in April 2009, an antigenically novel swine-origin H1N1 influenza A virus has spread throughout the world, causing the first influenza pandemic of the 21st century. Like most seasonal influenza viruses, this new virus is currently only associated with a mild self-limiting illness in the majority of people, although some populations (e.g. the young, and those with certain chronic health conditions) seem particularly susceptible to severe complications (Jain *et al.*, 2009).

We have begun to determine the effect that acquired mutations may have on the replication and pathogenicity of future circulating 2009 H1N1 virus strains. Here, we concentrate on the viral NS1 protein, which performs a number of functions to facilitate efficient virus propagation and is considered a major virulence factor (reviewed by Dundon & Capua, 2009; Hale *et al.*, 2008b). Specifically, NS1 blocks the activation of RIG-I, thereby inhibiting the interferon-induction signalling cascade in infected cells (Gack *et al.*, 2009; Guo *et al.*, 2007; Mibayashi *et al.*, 2007; Opitz *et al.*, 2007). In addition, most (but not all) NS1 proteins can block the global post-transcriptional processing of cellular mRNAs, a second mechanism that probably contributes to limiting host antiviral responses (Kochs *et al.*, 2007; Nemeroff *et al.*, 1998; Noah *et al.*, 2003; Twu *et al.*, 2007). NS1 also has well-characterized roles in: (i) inhibiting specific antiviral effector proteins such as 2′-5′-oligoadenylate synthetase (Min & Krug, 2006) and dsRNA-activated protein kinase (Li *et al.*, 2006; Min *et al.*, 2007); (ii) modulating host apoptosis (Schultz-Cherry *et al.*, 2001; Zhirnov *et al.*, 2002); (iii) stimulating phosphoinositide 3-kinase signalling (Hale *et al.*, 2006); and (iv) regulating viral RNA/protein synthesis (Burgui *et al.*, 2003; Min *et al.*, 2007).

There has been a great deal of work aimed at correlating virulence with strain-specific differences in the functions of NS1. In this study, we examined the C-terminal portion of NS1 beyond residue 202, which appears to be intrinsically disordered (Hale *et al.*, 2008a), and varies in length depending upon the viral strain [total NS1 length can be 202–237 residues (Dundon & Capua, 2009)]. This flexible tail has been implicated in the interaction of NS1 with poly(A)-binding protein II (Li *et al.*, 2001) and host importins (Melen *et al.*, 2007). Furthermore, the final four C-terminal residues of many 230 aa long avian influenza A

virus NS1 proteins constitute a consensus PDZ domain ligand (residues 227–230, e.g. ESEV or EPEV), which unlike their human virus counterpart sequences have the potential to bind to a plethora of PDZ domain-containing proteins (Obenauer *et al.*, 2006). Residues 212–217 of most avian influenza A virus NS1 proteins also constitute a class II SH3-binding motif (Finkelstein *et al.*, 2007), which displays remarkable binding specificity for the N-terminal SH3 domains of the Crk/CrkL signalling adaptor proteins (Heikkinen *et al.*, 2008). The functional significance of both these motif-based NS1–host interactions has yet to be fully determined. Nevertheless, presence of a PDZ domain ligand motif in NS1 appears to enhance viral pathogenicity, at least in a mouse model (Jackson *et al.*, 2008).

The NS1 protein of the currently circulating 2009 pandemic H1N1 virus is only 219 aa long, and thus lacks any potential C-terminal PDZ domain ligand motif. In addition, glutamic acid at residue 217 in this NS1 protein disrupts the class II

SH3-binding consensus sequence, ^{212}PxxPxK^{217}. The NS segment of the 2009 H1N1 virus is derived from the North American 'classical' swine H1N1 lineage, the precursor of which is related to the 1918 pandemic H1N1 virus. Sequence alignment of representative 'classical' swine H1N1 virus NS1 protein C-terminal tails since 1930 reveals that the ^{212}PxxPxK^{217} motif was present and maintained in the swine virus population until around the mid-1950s, after which glutamic acid replaced the lysine at position 217 (Fig. 1). Furthermore, NS1 was maintained at a length of 230 aa until the mid-1960s, when introduction of a stop codon resulted in truncation at position 219 (Fig. 1). Both of these changes have subsequently been retained in the 'classical' swine H1N1 lineage until the present day, and have now emerged with the novel 2009 H1N1 virus into the human population. Given that human seasonal influenza A virus NS1 proteins are generally 230 aa in length, and many have lysine at position 217 [albeit commonly without Crk/CrkL binding due to substitution of threonine for proline at 215
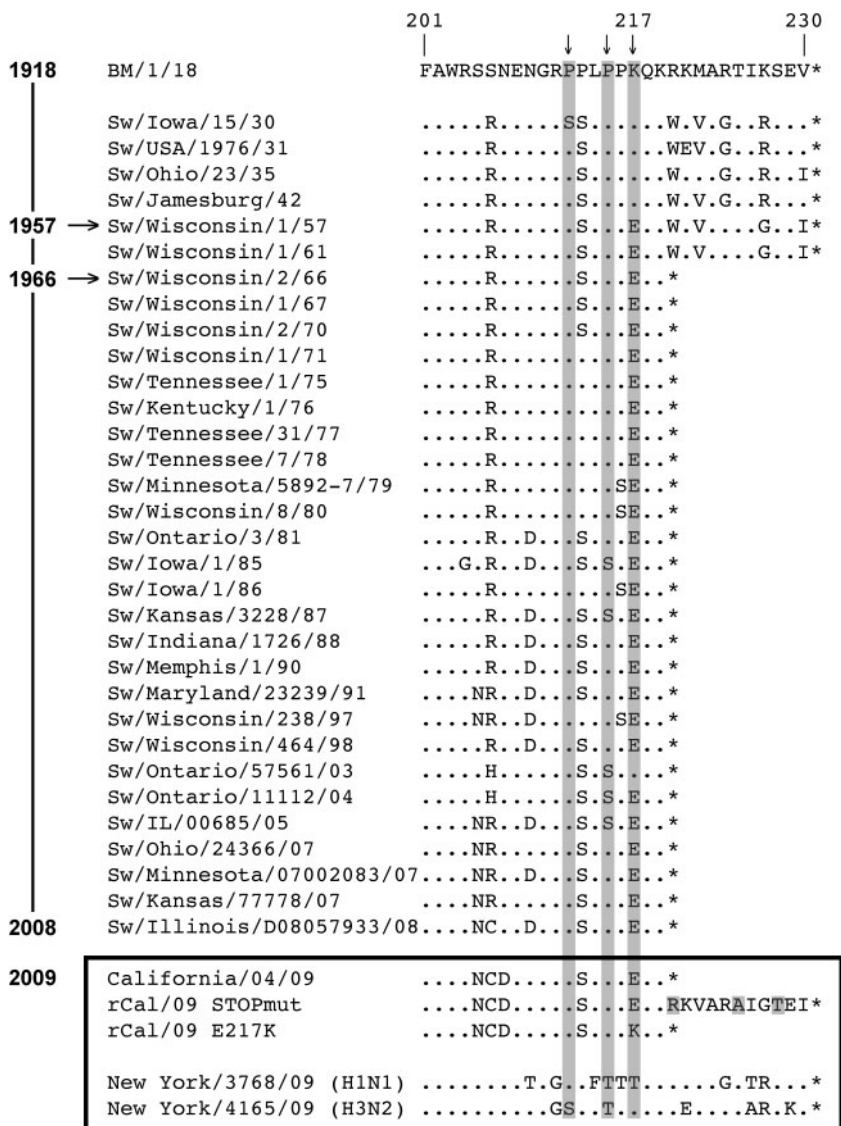

Fig. 1. Evolution of the 'classical' swine H1N1 influenza A virus NS1 protein C-terminal tail. Alignment of sequences derived from the C terminus (residues 201–230) of NS1 proteins encoded by the 1918 H1N1 influenza A virus (A/Brevig Mission/1/18; BM/1/18), representative North American 'classical' swine H1N1 viruses (1930–2008), and the 2009 pandemic H1N1 virus (A/California/04/09; Cal/09). The alignment is rooted with BM/1/18. Identity (.), Crk/CrkL-binding residues (↓ and grey shading) and stop codons (*) are indicated. The sequences of rCal/09 mutant viruses generated in this study are shown (black box). Highlighted aa (grey shading) in the rCal/09 STOPmut extended sequence indicate residues not observed in the 'classical' swine H1N1 lineage that are found in the longer NS1 protein. Such changes are due to natural 'silent' mutations in the previously non-coding sequence. For reference, the NS1 C-terminal tail sequences of two recent human seasonal H1N1 and H3N2 influenza A virus isolates are shown (black box).

(Finkelstein *et al.*, 2007; Heikkinen *et al.*, 2008), Fig. 1], we tested the effect of making these individual changes on the replication, pathogenicity and transmission of a prototype 2009 pandemic H1N1 influenza virus isolate, A/California/04/09 (Cal/09).

As described previously (Hai *et al.*, 2010), a plasmid-based reverse genetics system was used to generate recombinant mutant Cal/09 viruses expressing NS1 with either a C-

terminal extension of 11 aa, or an E217K aa substitution (Fig. 1, lower box). As required by the Mount Sinai School of Medicine institutional biosafety committee, work involving these viruses was carried out in either a USDA- and CDC-approved BSL3+ containment laboratory, or a dedicated BSL2 laboratory with personnel adhering to strict BSL3 working practices. Both mutant viruses were created by changing a single nucleotide in the NS segment, neither of which affected the NS2/NEP open reading frame
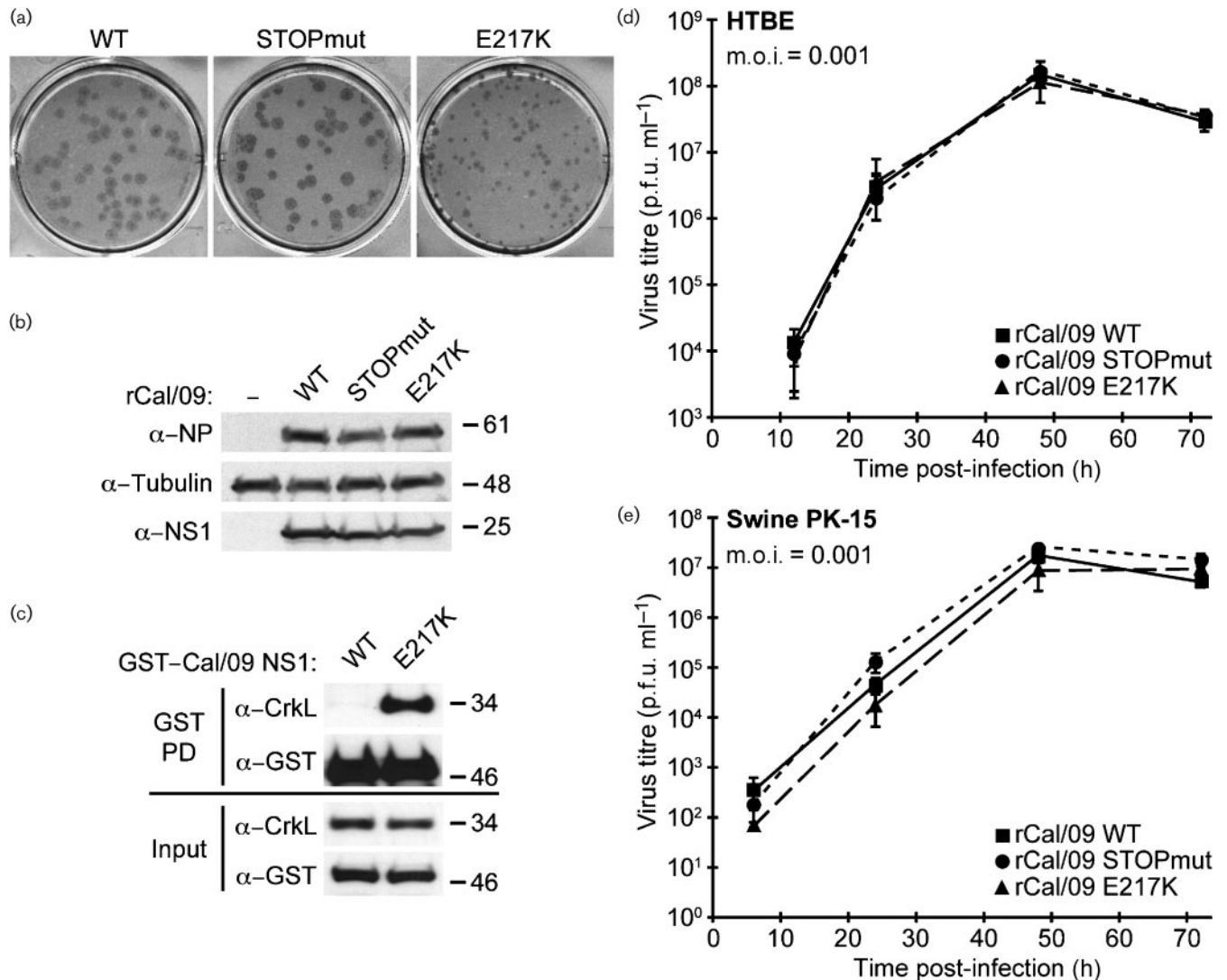

Fig. 2. *In vitro* characterization of the rCal/09 STOPmut and E217K viruses. (a) Plaque phenotype of rCal/09 WT, rCal/09 STOPmut and rCal/09 E217K viruses in MDCK cells. (b) Western blot determination of viral NP and NS1 protein expression in MDCK cells infected for 12 h with the rCal/09 WT, rCal/09 STOPmut or rCal/09 E217K viruses (m.o.i. of 5 p.f.u. per cell). Tubulin acted as a loading control. Molecular mass markers (kDa) are indicated. (c) Interaction of cellular CrkL with Cal/09 NS1. GST-tagged NS1 (WT or E217K Cal/09) was expressed in 293T cells and pull-down (PD) assays performed using glutathione–agarose beads. Following SDS-PAGE, precipitated proteins were detected by Western blot by using specific anti-GST and anti-CrkL antibodies. These proteins were also detected in the soluble lysate prior to pull-down ('Input'). Molecular mass markers (kDa) are indicated. (d) Multicycle growth analysis of rCal/09 WT, rCal/09 STOPmut and rCal/09 E217K viruses in primary differentiated HTBE cells, and the swine cell line PK-15 (e). Data points show mean values from triplicate experiments and error bars represent SD.

(ORF). For the extended NS1 (STOPmut), codon 220 was changed from TGA to CGA, thus replacing the existing stop codon with an arginine codon (as found in most human influenza A virus NS1 proteins). This extends the NS1 ORF to 230 codons, at which point a natural stop codon is present. The aa sequence of this extended tail is highly similar to that of previously circulating 'classical' swine H1N1 influenza viruses in the 1960s, albeit with three changes (Fig. 1, lower box: W220R, T225A and S228T). Like its 'classical' swine predecessors, the four C-terminal residues of this new NS1 tail do not make up a consensus PDZ domain ligand motif.

Genotypes of the rescued, plaque-purified rCal/09 viruses were confirmed by direct sequencing of the RT-PCR products of the entire NS segments. *In vitro* characterization of the engineered rCal/09 STOPmut virus revealed that both this and the rCal/09 wild-type (WT) virus had similar plaque phenotypes in Madin–Darby canine kidney (MDCK) cells (Fig. 2a). Interestingly, the rCal/09 E217K virus displayed a small-plaque phenotype (Fig. 2a). Western blot analysis confirmed that relative levels of viral NS1 and NP proteins were identical in infected MDCK cells for all three viruses, suggesting no gross differences in mutant NS1 protein expression or stability [m.o.i. of 5 p.f.u. per cell, 12 h post-infection; Fig. 2(b) and data not shown]. We were unable to resolve any migrational differences between the 219 aa WT NS1 protein and the 230 aa STOPmut NS1 protein by SDS-PAGE (Fig. 2b). However, we confirmed that the single E217K aa change was able to confer CrkL-binding activity to the Cal/09 NS1 protein. Glutathione *S*-transferase (GST) fused to either WT Cal/09 NS1 or Cal/09 NS1-E217K was expressed in human embryonic kidney (293T) cells, and protein lysates were subjected to GST pull-down assays. Analysis of precipitated proteins by Western blot revealed that endogenous CrkL was only co-precipitated with the Cal/09-NS1 E217K mutant, and not with WT Cal/09 NS1 (Fig. 2c, upper panel). Analysis of whole-cell lysates confirmed similar expression levels of the input proteins (Fig. 2c, lower panel). We also assessed replication of the three rCal/09 viruses in primary differentiated human tracheobronchial epithelial cells (HTBEs) and the swine cell line PK-15. As shown in Fig. 2(d and e) both the rCal/09 STOPmut and E217K viruses grew with similar kinetics to WT during multicycle growth analyses in both human and swine cell-types (input m.o.i. of 0.001 p.f.u. per cell).

To test whether the increased length of NS1 or its ability to bind Crk/CrkL would affect *in vivo* replication and/or pathogenicity of the 2009 H1N1 virus, we initially compared the rCal/09 WT, rCal/09 STOPmut and rCal/09 E217K viruses in the highly susceptible DBA/2J mouse model (Boon *et al.*, 2009; Srivastava *et al.*, 2009). All animal procedures were performed in accordance with the Institutional Animal Care and Use Committee guidelines of Mount Sinai School of Medicine, and were carried out in a BSL3 containment laboratory. Seven-week-old female DBA/2J mice (The Jackson Laboratory, ME, USA) were anaesthetized using ketamine–xylazine, infected with 10^5 p.f.u. of each virus (or PBS alone, 50 µl intranasally, $n=5$), and body weights were monitored for 14 days. The average maximum body weight loss was similar for all three virus-infected groups (Table 1, upper panel), whilst mice in the PBS-treated group did not lose any weight. In parallel, lungs were excised from euthanized mice and used to determine viral titres. All three viruses appeared to reach similar mean peak infectious titres at day 2, and no differences between virus titres were observed at day 4 (Table 1, upper panel). However, the rCal/09 STOPmut virus seemed to be cleared marginally faster than both the WT and E217K viruses, as evidenced by viral titres on day 7 (Table 1, upper panel).

Table 1. *In vivo* characterization of the rCal/09 STOPmut and E217K viruses in mouse and ferret models

DBA/2J mice 10^5 p.f.u. per mouse	Maximum weight loss (% ± SD)*	Day 2 lung titre ($\log_{10}$ ± SD)†	Day 4 lung titre ($\log_{10}$ ± SD)†	Day 7 lung titre ($\log_{10}$ ± SD)†
rCal/09 WT	16.1 ± 2.8	6.8 ± 0.2	5.8 ± 0.1	4.9 ± 0.2
rCal/09 STOPmut	16.0 ± 3.3	6.7 ± 0.2	5.7 ± 0.2	3.9 ± 0.5
rCal/09 E217K	13.9 ± 5.9	6.5 ± 0.1	5.4 ± 0.1	4.9 ± 0.5

Ferrets 10^6 $TCID_{50}$ per ferret	Direct and aerosol transmission‡	NT titre ($\log_{10}$)§	Tracheal titre ($\log_{10}$)§	Lung titre ($\log_{10}$)§
rCal/09 WT	(2/2) and (2/2)	6.1 (5.9, 6.2)	3.3 (3.2, 3.4)	4.3 (3.4, 5.2)
rCal/09 STOPmut	(2/2) and (2/2)	5.9 (5.7, 6.0)	2.9 (2.4, 3.5)	3.9 (3.4, 4.4)
rCal/09 E217K	(2/2) and (2/2)	4.8 (4.7, 4.9)	2.8 (2.3, 3.4)	3.4 (3.4, 3.5)

*Body weights of infected mice were determined daily. Mean maximum body weight loss (typically days 7 or 8 post-infection) is indicated ($n=5$).
†Mean lung titres from three mice per group.
‡Transmission events were scored after determining daily nasal wash titres from infected ferrets ($n=2$), direct contact ferrets ($n=2$) and aerosol (large- and small-respiratory droplet) contact ferrets ($n=2$).
§Mean viral titres in nasal turbinates (NT), trachea and lungs were determined on day 5 post-infection ($n=2$). Results from individual replicates are shown in parentheses.

Finally, we studied replication of the rCal/09 WT, rCal/09 STOPmut and rCal/09 E217K viruses in a ferret model. All ferret procedures were performed in accordance with the Institutional Animal Care and Use Committee guidelines of the University of Maryland, and were carried out in a USDA-approved BSL3+ containment laboratory. The basic transmission study scheme consisted of duplicate groups of three 6–8-month-old female Fitch ferrets (Triple F Farms, PA USA): one infected intranasally with 10^6 $TCID_{50}$ of virus in PBS (600 μl per nostril), one infected by direct contact and one infected by respiratory droplet contact. Nasal washes were collected daily and viral titres were determined in order to assess transmission events. The experimental details were essentially as described previously (Sorrell et al., 2009). Although we noted slightly higher virus shedding with the WT rCal/09 virus (data not shown), all three viruses transmitted equally efficiently to other ferrets by both direct contact and respiratory droplet routes (Table 1, lower panel). The amount of infectious virus present in different parts of the ferret respiratory tract was also determined at day 5 post-infection. All viruses reached similar titres in the trachea and lungs; however, the rCal/09 E217K virus grew to titres approximately 10-fold lower than both WT and STOPmut viruses in nasal turbinates (Table 1, lower panel).

In summary, we have investigated the potential effects that specific NS1 mutations may have on the replication and virulence of the emergent 2009 pandemic H1N1 virus. We concentrated on the C-terminal tail of this NS1 protein due to its uniquely short length (as compared with most human influenza A virus strains), and its previously published possible associations with virulence (Heikkinen et al., 2008; Jackson et al., 2008; Obenauer et al., 2006). Although human influenza A virus NS1 proteins do not generally have a consensus Crk/CrkL-binding motif, we sought to assess the impact of the E217K aa substitution as it has already arisen naturally in a human 2009 H1N1 virus isolate (A/Wisconsin/629-D00022/09), and Crk/CrkL binding is a feature of both highly pathogenic avian influenza A viruses and the devastating 1918 pandemic virus (Heikkinen et al., 2008). Overall, our data indicate that neither the C-terminal extension of NS1 nor the E217K substitution (both resulting from single nucleotide changes) has a major effect on replication, virulence or transmissibility of the novel 2009 H1N1 virus. Thus, the possible future detection of these viruses in the human population may not be an immediate cause for concern.

From a basic science perspective, our work also directly addresses the role of Crk/CrkL binding and natural NS1 tail length variation on influenza A virus replication and pathogenicity. Although we saw limited impact in our systems, we do not exclude the possibility that biological effects may be more apparent with different virus strains or host species. Indeed, the current 2009 H1N1 virus genetic constellation may already be optimized to function so efficiently that gain of new NS1 activities disrupts a subtle virus–host equilibrium, thus leading to slight attenuation of the virus. This hypothesis is also borne out by the observations of others, who have additionally been unable to experimentally increase replication of the 2009 H1N1 virus by reverse genetics methods (Hai et al., 2010; Herfst et al., 2010). Clearly, it will be worth monitoring the natural evolution of this swine-origin virus, along with its unique NS1 protein, now that it is under human selection pressures.

Note Added in Proof

A naturally-occurring 2009 pandemic H1N1 virus (A/Australia/79/2009) expressing a 230 aa long NS1 protein has recently been isolated from a human patient. The 11 aa NS1 extension is the result of a change in codon 220 from TGA to TGG, thus encoding the swine consensus residue W220. This single nucleotide change also results in an amino-acid substitution in NS2/NEP.

Acknowledgements

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