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Adaptive Mutations That Occurred during Circulation in Humans of H1N1 Influenza Virus in the 2009 Pandemic Enhance Virulence in Mice

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ABSTRACT
During the 2009 H1N1 influenza pandemic, infection attack rates were particularly high among young individuals who suffered from pneumonia with occasional death. Moreover, previously reported determinants of mammalian adaptation and pathogenicity were not present in 2009 pandemic H1N1 influenza A viruses. Thus, it was proposed that unknown viral factors might have contributed to disease severity in humans. In this study, we performed a comparative analysis of two clinical 2009 pandemic H1N1 strains that belong to the very early and later phases of the pandemic. We identified mutations in the viral hemagglutinin (HA) and the nucleoprotein (NP) that occurred during pandemic progression and mediate increased virulence in mice. Lethal disease outcome correlated with elevated viral replication in the alveolar epithelium, increased proinflammatory cytokine and chemokine responses, pneumonia, and lymphopenia in mice. These findings show that viral mutations that have occurred during pandemic circulation among humans are associated with severe disease in mice.

IMPORTANCE
In this study, novel determinants of 2009 pandemic H1N1 influenza pathogenicity were identified in the viral hemagglutinin (HA) and the nucleoprotein (NP) genes. In contrast to highly pathogenic avian influenza viruses, increased virulence in mice did not correlate with enhanced polymerase activity but with reduced activity. Lethal 2009 pandemic H1N1 infection in mice correlated with lymphopenia and severe pneumonia. These studies suggest that molecular mechanisms that mediate 2009 pandemic H1N1 influenza pathogenicity are distinct from those that mediate avian influenza virus pathogenicity in mice.

The first pandemic of the 21st century was caused by a novel influenza A virus strain of the H1N1 subtype that contained gene segments from both North American and Eurasian swine lineages (1–3). In the beginning, the 2009 H1N1 influenza pandemic was considered to be relatively mild as the majority of cases underwent an uncomplicated or even an asymptomatic infection course. However, this was partially revoked since infection attack rates were highest among the younger age groups, in contrast to seasonal influenza, where mostly the elderly are affected (4–7). During the pandemic in 2009, a disproportionately high number of young adults were hospitalized due to pneumonia and eventually died (6–9). Retrospective modeling estimates that during the first 12 months of the pandemic, approximately 80% of the overall 201,200 respiratory and additional 83,300 cardiovascular deaths occurred in people younger than 65 years (10). This age-specific mortality pattern among younger individuals has been captured by the “years-of-life-lost” metric (11) as a more accurate parameter to measure pandemic burden. In the United States, the number of years of life lost within the first month of the pandemic was estimated to range between the impact of the more virulent H3N2 influenza epidemics and that of the 1968 Hong Kong pandemic (12).

Subsequently, it was postulated that 2009 pandemic H1N1 (2009 pH1N1) influenza viruses must contain virulence markers that contributed to disease severity among healthy individuals. Viral sequence analysis highlighted that 2009 pH1N1 influenza viruses do not harbor previously identified markers of mammalian adaptation and/or pathogenesis, suggesting that novel and yet still largely unrecognized sites must have contributed to severe disease outcome in humans (3). This was further supported by studies using various animal models where 2009 pH1N1 influenza virus infection was more severe in mice, ferrets, and nonhuman primates compared to seasonal influenza viruses (2, 13–15). In C57BL/6J mice (15, 16) and the nonhuman primate model (14), even differences in virulence were observed among the 2009 H1N1 virus variants that circulated during the pandemic. These data support the concept that 2009 pH1N1 influenza viruses not only possess previously unrecognized markers predictive of human adaptation and pathogenesis but also differ in their pathogenic potential.

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In this study, we sought to identify 2009 pH1N1 markers of mammalian pathogenicity using the previously proposed C57BL/6J mouse model (15). Therefore, we have used clinical isolates representative of different pandemic phases.

MATERIALS AND METHODS

Cells and viruses. MDCK (Madin-Darby canine kidney) cells were grown in minimal essential medium (MEM) (PA), while HEK293T (human embryonic kidney) and A549 (human lung carcinoma) cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (PA). Both media were supplemented with 10% fetal calf serum (FCS) (PA), 1% glucose (PA), and 1% penicillin-streptomycin (PA). The 2009 pH1N1 wild-type viruses A/Hamburg/05/09 (abbreviated “HH05”) and A/Hamburg/NY1580/09 (abbreviated “HH15”) were isolated from pharyngeal swabs of infected patients during the influenza pandemic in 2009 as described before (15).

Recombinant 2009 pH1N1 viruses. Recombinant 2009 pH1N1 viruses were generated by reverse genetics using the pHW2000-based 8-plasmid system as described previously (17). Mutations in the single-point mutant (SPM) viruses were introduced by site-directed mutagenesis with a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol. Subsequently, recombinant viruses were sequenced to verify introduced amino acid substitutions and to exclude additional mutations.

Ethics statement. Mouse experiments were performed in the institutional facilities according to the guidelines of the German Animal Protection Law. Animal protocols were approved by the relevant German authorities.

Animal experiments. C57BL/6J mice were obtained from Charles River or Harlan Laboratories. Female mice 4 to 8 weeks old were anesthetized with 100 mg/kg ketamine–10 mg/kg xylazine and inoculated intranasally with 50 μl virus diluted in phosphate-buffered saline (PBS). Control groups received PBS. Animals were observed for 14 days for weight loss and survival. The mouse 50% lethal dose (MLD50) was assessed by infecting mice with serial 10-fold virus dilutions and calculated as described previously (18). For lung histology, three C57BL/6J mice were infected with 105 PFU of the respective virus. On day 6 postinfection (p.i.), animals were sacrificed and lungs removed. Additionally, on days 3 and 6 p.i. three animals per time point were sacrificed, lungs were removed, and virus titers were determined by plaque assays.

In situ hybridization. Virus RNA in tissues was detected using single-stranded 35S-labeled viral RNA probes, which were synthesized from a plasmid with HindIII or KpnI, respectively, and subsequent T7 RNA polymerase transcription produced an antisense RNA probe suitable for detection with the Life Technologies Human Cytokine Magnetic 25-Plex Panel kit according to the manufacturer’s instructions. The cytokine levels shown represent average results from two independent experiments measured in duplicate.

Automated lymphocyte cell counts. C57BL/6J mice were infected with 105 PFU of the 2009 pH1N1 viruses, and blood samples were taken on day 3 p.i. Lymphocyte counts were analyzed in automated blood cell counts on whole blood with poch 1000 Diff (Sysmex). Recommended settings and calibrations for mouse strain-specific hematometry were set according to the manufacturers’ protocols.

Prevalence of HH15-specific mutations. Determination of polymorphism frequencies was based on amino acid sequences for the proteins PB2, PA, NP, hemagglutinin (HA), NA, and NS1. Amino acid sequences for 2009 pH1N1 isolates from 2009 to 2014 with respective sampling times (year/month) were downloaded from the GISAID (20) database (see Table S1 in the supplemental material). The numbers of isolates for each segment were as follows: PB2, 3,356; PA, 3,202; NP, 3,319; HA, 9,728; NA, 11,017; and NS1, 3,408. For each protein, the analysis pipeline was applied: first duplicate isolates for same strains and then isolates with missing sampling times were removed. After alignment of the sequences with MUSCLE (21) and manual curation of the alignments with JalView (22), the mutations were binned by their isolation dates into four pandemic phases, adopting definitions used in the literature (16, 23). For the HA protein, the H1 numbering based on the sequence was used (42). Finally, the frequencies of the polymorphisms characteristic for either of the 2009 pH1N1 viruses HH05 or HH15 were determined for each bin.

RESULTS

Mutations in HA and NP increase 2009 pH1N1 virulence in C57BL/6J mice. We have previously shown that two clinical strains isolated at very early (April 2009; named “HH05”) and later (June 2009; named “HH15”) time points of the pandemic display differential virulence in C57BL/6J mice. While HH05 is low pathogenicity (MLD50, 5.2 log10 PFU), HH15 is approximately 50 times more virulent (MLD50, 3.5 log10 PFU) in C57BL/6J mice. Both viruses differ by 12-amino-acid exchanges in the genes encoding the viral surface proteins, the ribonucleoprotein complex, and the nonstructural protein 1 (15). In order to identify which of these amino acid substitutions present in the virulent HH15 strain (PB2, K340N and R526K; PA, I118V and L581M; NP, V100I, I133L, and I373T; HA, S202T; NA, T321I, V106L, and N248D; and NS1, I123V) are responsible for enhanced virulence, we have generated single-gene reassortant (SGR) and SPM viruses in the HH05 genetic backbone. First, we confirmed that the recombinant 2009 pH1N1 influenza virus strains HH05rec and HH15rec show differential virulence in the C57BL/6J mouse model comparable to that of their parental wild-type strains reported before (Fig. 1). Consistent with previous reports on the clinical isolates (15), HH05rec is low pathogenicity, with an MLD50 of >5 log10 PFU, whereas HH15rec is about 50 times more pathogenic, with an MLD50 of 3.2 log10 PFU (Fig. 1). Among the SGRs tested, only those containing HH15-HA or -NP led to enhanced virulence, while the PB2, NA, and NS genes of HH15 did not significantly affect pathogenicity in mice. The MLD50 of HH05-NP/HH15, with 3.2 log10 PFU, and HH05-HA/HH15, with 3.6 log10 PFU, are comparable to the high virulence of the HH15rec and the parental HH15 strain. The “6 + 2” combined reassortant virus HH05-PB2/PA/NP/HH15 with the HH15-specific PB2, PA, and NP genes as well as the “6 + 2” combined virus HH05-HA/NA/HH15 with HH15-specific HA and NA genes were both highly pathogenic in C57BL/6J mice (MLD50, 3.5 log10 PFU and 3.8 log10 PFU) (Fig. 1). Since the NP of HH15 differs from HH05 by three amino acids, we
Virulence-increasing mutations in HA and NP cause alveolar destruction and inflammation in mice. The main cause of death among 2009 pH1N1-infected humans was the acute respiratory distress syndrome (ARDS). A particular hallmark of ARDS is alveolar destruction and inflammation (24). Here, we analyzed the impact of the virulence-increasing mutations in HA and NP of HH15 on lung pathology in infected C57BL/6J mice.

Virulence-increasing mutations in HA and NP cause alveolar destruction and inflammation in mice. The main cause of death among 2009 pH1N1-infected humans was the acute respiratory distress syndrome (ARDS). A particular hallmark of ARDS is alveolar destruction and inflammation (24). Here, we analyzed the impact of the virulence-increasing mutations in HA and NP of HH15 on lung pathology in infected C57BL/6J mice (Fig. 2). In HH15-infected lungs, significantly more virus RNA-positive cells were detected than in HH05-infected mice (Fig. 2B). Moreover, the grade of alveolar destruction and monocellular cell infiltration were similarly high in SGR HH05-NPV100I-infected mice, as observed upon HH15 infection. However, the number of virus-positive cells in SGR HH05-NPV100I-infected mice was more comparable to those observed with HH05rec (Fig. 2D and I). SGR HH05-NPV100I-infected mice revealed diffuse alveolar destruction compared to the HH05-infected groups. Viruses harboring NP V100I showed the largest amounts of viral RNA-positive cells comparable to HH15 in mice. Thus, grade of viral replication in the alveoli as well as alveolar inflammation correlates with increased 2009 pH1N1 virulence in C57BL/6J mice.

Virulence-increasing mutations in NP do not affect viral polymerase activity but increase viral replication in human lung cells. We next addressed the question of whether increased virulence and viral replication in mice are due to an increase in viral polymerase activity, as shown before for highly pathogenic avian influenza viruses that were either isolated from humans or adapted to mammals (25). Moreover, we wanted to know whether the surface-exposed mutations in NP (Fig. 3A) might contribute to a potentially altered viral polymerase activity in mammalian cells. Therefore, we reconstituted the viral ribonucleoprotein (RNP) complex in human lung cells and measured viral polymerase activity using a reporter gene assay as described before (26). Surprisingly, HH15 polymerase activity was significantly reduced compared to HH05 polymerase activity in human lung cells (P < 0.001) (Fig. 3B). Introduction of HH15-PB2 significantly reduced HH05 polymerase activity (P < 0.001), while the PA and NP proteins of HH15 did not alter HH05 polymerase activity (Fig. 3B). Next, we analyzed whether reduced HH15 polymerase activity correlates with diminished viral replication in mammalian cells. Therefore, we infected human lung cells with HH05rec, HH15rec, and the SGR and SPm viruses and determined virus titers at various time points after infection. In general, HH15rec showed a slightly increased replication compared to HH05rec at 96 h p.i. (Fig. 3C). This elevated viral replication was mainly mediated by HH15-NP, while HH15-HA had either no effect or even correlated with decreased viral growth at 72 h p.i. (Fig. 3C). Viral growth of SPm viruses revealed that the NP-I133L mutation is mainly responsible for increased viral replication in the lung epithelial cells. Moreover, these findings suggest that viral polymerase activity is not a direct correlate of HH15 replication in human lung cells.

Virulence-increasing mutations in NP mediate elevated pro-inflammatory cytokine and chemokine responses in human lung cells. Next, we analyzed whether HH15-specific mutations in NP affect the cellular innate response known to be an important determinant of influenza virus pathogenicity. Therefore, we analyzed the expression levels of a panel of cytokines, namely, interferon alpha (IFN-α), interleukin-6 (IL-6), IL-8, and monocyte chemoattractant protein 1 (MCP-1), in human lung cells infected with recombinant 2009 pH1N1 influenza viruses. No significant differences in the analyzed cytokine expression levels were observed with recombinant 2009 pH1N1 influenza viruses as compared to that in HH05rec infection, significantly more viral RNA-positive cells were detected compared to other SPm viruses or the parental HH05rec strain (Fig. 21). The SPm HH05-NP I133I- or HH05-NP I373T-infected animals presented a high grade of alveolar destruction with some virus-positive cells. However, the highest grade of viral replication was detected when all three NP mutations were combined in SGR HH05-NP I133I I373T-infected mice, resulting in similar high replication kinetics to HH15 (Fig. 2I). In all cases, viral RNA-positive cells were mostly observed in alveolar cells rather than the bronchial epithelium (Fig. 2B to H).

Overall, HH15rec-infected mice showed an increased grade of viral RNA-positive cells as well as alveolar inflammation and destruction compared to the HH05-infected groups. Viruses harboring mutations in HA (S202T) and NP (I133L and I373T) led to a particularly high grade of alveolar inflammation, while SPm harboring NP V100I showed the largest amounts of viral RNA-positive cells comparable to HH15 in mice. Thus, grade of viral replication in the alveoli as well as alveolar inflammation correlates with increased 2009 pH1N1 virulence in C57BL/6J mice.

**FIG 1** Virulence of recombinant 2009 pH1N1 influenza viruses in C57BL/6J mice. C57BL/6J mice were intranasally infected with serial 10-fold dilutions (10^6 to 10^0 PFU) of the recombinant (rec) 2009 pH1N1 viruses or the parental HH05rec strain (Fig. 1). The SPm HH05-NP I133I- or HH05-NP I373T-infected animals presented a high grade of alveolar destruction with some virus-positive cells. However, the highest grade of viral replication was detected when all three NP mutations were combined in SGR HH05-NP I133I I373T-infected mice, resulting in similar high replication kinetics to HH15 (Fig. 2I). In all cases, viral RNA-positive cells were mostly observed in alveolar cells rather than the bronchial epithelium (Fig. 2B to H).
served between HH05rec- and HH15rec-infected cells at 3 and 6 h p.i. (Fig. 4). IFN-α expression levels were significantly increased upon infection with the SPM viruses HH05-NPV100I and HH05-NPI373T at 3 h p.i. compared to HH05rec (Fig. 4A). IL-6 expression was significantly increased in HH05-NPV100I and HH05-NPI373T-infected cells compared to HH05-infected cells at 3 and 6 h p.i. (Fig. 4B). The HH05-NPI373T SGR also showed increased IL-6 expression levels at 6 h p.i. IL-8 expression levels were significantly increased upon infection with HH05-NPI373T in relation to other infections at 3 h p.i. (Fig. 4C). Similarly, the highest expression levels of MCP-1 were detected in cells infected with HH05-NP100I and HH05-NP133L, and 3 h p.i. (Fig. 4D).

Overall, virulence-increasing mutations in NP showed the highest IFN-α, IL-8, and MCP-1 expression levels in infected human lung cells.

Virulence-increasing mutations in NP mediate lymphopenia in mice. It was previously reported that lymphopenia is a hallmark of severe 2009 pH1N1 (15) and avian (27) influenza virus infection in mice. Here, we analyzed whether virulence-increasing mutations in HA and NP might affect lymphocyte counts and thus contribute to disease severity in mice. Therefore, we analyzed the lymphocyte counts in the blood of infected mice collected on day 3 p.i. (Fig. 5). Compared to uninfected control groups, infection with HH05rec or HH15rec as well as the SGR and SPM viruses led to generally reduced lymphocyte counts (Fig. 5). However, lymphocytes were most strongly reduced upon infection with HH15rec or the SPM viruses HH05-NPV100I, HH05-NPI133L, and HH05-NPI373T compared to mice infected with HH05 or SGR viruses. These data show that lymphopenia strongly correlates with increased virulence in mice. Moreover, severe lymphopenia was mediated by 2009 pH1N1 viruses harboring NP V100I, I133L, and I373T mutations that are associated with increased virulence in mice.

Prevalence of HH15-specific amino acid substitutions among humans. In order to assess the prevalence of HH15-specific amino acids in human 2009 pH1N1 isolates, we have per-
formed a database analysis (Table 1). The obtained amino acid frequencies were then differentiated according to previously defined pandemic phases (16, 23). In general, HH15-specific amino acids were less frequent in the early pandemic phase and became most prevalent with frequencies from 52.16% to 100%, with the exception of PB2 340N and PA 118V at the postpandemic phase. Interestingly, with the two exceptions mentioned above, the HH05-specific amino acids almost disappeared from circulation among the human population in the postpandemic phase. These findings are in line with previous reports that the HH15 strain is a representative of 2009 pH1N1 influenza viruses isolated during later phases (15, 16).

DISCUSSION

The 2009 H1N1 influenza pandemic was associated with a considerable health burden in terms of hospitalization and deaths, especially among those under 65 years of age (4–6, 10, 12). However, previously reported molecular markers of adaptation and pathogenicity in humans described for H5N1, H7N7, H7N9, or 1918 pandemic influenza viruses were absent in 2009 pH1N1 strains (3, 25).

Therefore, we aimed to identify previously unrecognized viral markers that might have been responsible for severe disease outcome during pandemic virus circulation in humans. Using the C57BL/6J mouse model described earlier (15), here we found single mutations in HA and NP that increase virulence of 2009 pH1N1 influenza viruses in mice. The low-pathogenicity HH05 strain used in this study belongs to the clade 1 lineage as one of the earliest and most homogenous clades that have emerged during the pandemic and probably circulated for approximately 2 to 6 weeks before its initial detection. In contrast, the more virulent HH15 strain belongs to the previously reported clade 7, which represented more than 30% of the 2009 pH1N1 strains that circulated later during the pandemic from April to July 2009 (28). The evolution of 2009 pH1N1 influenza viruses during circulation among humans from an initially mixed clade with limited geographic distribution to the predominance of clade 7 was postulated to have accounted for most of the pandemic burden worldwide (29).

Interestingly, two of the 2009 pH1N1 virulence-increasing mutations (HA S202T and NP V100I) present in clade 7 strains were less prevalent (0 to 10%) in the prepandemic period, while their prevalence continuously increased to 80 to 90% during later pandemic phases (June to December 2009) (23).

In our study, we further identified two additional mutations in NP (I133L and I373T) that mediate enhanced pathogenicity in mice. Combination of all three NP mutations, however, did not present additive effects in terms of virulence in mice suggesting that these single sites do not act synergistically. Similarly, NP mutations (I133L and I373T) became gradually prevalent from the pre- to postpandemic phases and are currently present in more than 90% of the currently circulating strains.

Increased HH15 virulence correlated with increased viral replication in the lung as measured by in situ hybridization since virus...
titers in lung homogenates (Fig. 6) have been shown not to represent a reliable parameter in C57BL/6J mice upon 2009 pH1N1 infection (15). Moreover, increased HH15 virulence correlated with severe alveolar damage, inflammation, and pneumonia in mice. These features are consistent with hallmarks of ARDS as the main cause of death among influenza patients (24). In the C57BL/6J mouse model, the increased viral replication was mediated by NP V100I, and elevated inflammation was mediated by HA S202T and NP I133L and I373T mutations, which together resulted in increased 2009 pH1N1 virulence. Surprisingly, this increased HH15 replication in the murine lung did not correlate with increased polymerase activity as shown for many avian influenza viruses that had infected humans (25). Instead, a reduced polymerase activity correlated with increased virulence in mice. Viral replication of the HH15 strain was nevertheless increased in human lung cells compared to that of HH05, suggesting that the replicative advantage is mediated by other mechanisms. Thus, measuring the polymerase activity alone might not necessarily reflect the virulence potential of 2009 pH1N1 viruses. It should also be noted that both strains used in this study harbor the adaptive PB2 590S and 591R positions reported to increase the polymerase activity of 2009 pH1N1 influenza viruses (30). The reduced HH15 polymerase activity compared to that of HH05 was mediated by mutations in PB2 (K340N and R526K). Since 2009 pH1N1 strains generally lack the PB2 human adaptive sites at position 627K (31) or 701N (26) known for avian influenza viruses, other alternative strategies seem to be used by 2009 H1N1 influenza viruses to overcome the reduced viral polymerase activity in order to achieve increased viral replication in mammalian cells.

The innate host response plays an important role in the outcome of acute infections (32). Here, 2009 pH1N1-infected human lung cells showed a general increase in IL-6 expression levels consistent with previous observations in severely ill patients (11, 33, 35). Moreover, induction of IFN-α, IL-8, and MCP-1 expression levels was mediated by mutations in NP strongly correlating with virulence in mice. However, no significant differences between HH05- and HH15-infected cells were detected. One possible explanation for this observation might be that other inflammatory cytokines contribute to HH15 virulence that are not directly re-

**FIG 4** Cytokine response in recombinant 2009 pH1N1-infected human lung cells. Human lung cells (A549) were either treated with PBS as a control (white bars) or infected with recombinant 2009 pH1N1 virus HH05rec (gray bars) or HH15rec (black bars), SGR virus HH05-HAHH15, or HH05-NPHH15 (gray bars with stripes), or SPM virus with HH15-specific amino acids in NP at positions 100I, 133L, and 373T in the HH05 background (HH05-NPVVHH15, HH05-NPI133L, or HH05-NPI373T) (gray bars with dots) at an MOI of 2. Cytokine levels were measured 3 and 6 h p.i. by multiplex bead assay. Here, we show those where significant differences were detected: interferon alpha (IFN-α) (A), interleukin-6 (IL-6) (B), IL-8 (C), and monocyte chemoattractant-protein 1 (MCP-1) (D). HH05-infected cells were set at 100% as a reference. Bars represent mean cytokine concentrations and standard deviations from two independent experiments measured in duplicate. Thus, the data sets comprise four individual measure points. Statistical analysis was performed using Student’s t test (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

**FIG 5** Lymphocyte counts in recombinant 2009 pH1N1 virus-infected C57BL/6J mice. C75BL/6J mice were either intranasally treated with PBS as a control or infected with 10^4 PFU of recombinant 2009 pH1N1 virus HH05rec or HH15rec, SGR virus HH05-HAHH15, or HH05-NPHH15 or SPM virus with HH15-specific amino acids in NP at positions 100I, 133L, and 373T in the HH05 background (HH05-NPVVHH15, HH05-NPI133L, or HH05-NPI373T). On day 3 p.i., blood samples were collected (n = 5 to 11) and lymphocytes were detected using an automated blood cell count. Controls were set at 100%. Means of individual analyses and standard deviations are shown. Statistical analysis was performed using Student’s t test (*, P < 0.05; ***, P < 0.001).
flected by the SGR or SPM viruses used in this study. However, single mutations in HA and NP were sufficient to mediate increased virulence in mice. Moreover, proinflammatory cytokine/chemokine responses mediated by NP human adaptive mutations correlated with alveolar inflammation observed in mice. Severe lymphopenia was an additional strong correlate of lethal 2009 pH1N1 infection in mice, consistent with previous reports of fatal disease (15, 27, 36).

Virulence-increasing mutations in HA and NP are located on the surface of the viral proteins. The HA S202T mutation is located in the globular head domain. Although this mutation is not directly located in the receptor binding pocket, it is known that the globular head might indirectly influence its receptor binding on host cells (37). Thus, future studies are required to analyze whether the HA mutation in the late-phase strains might affect receptor binding and then, as a consequence, influenza virus transmission among mammals. The mutation V100I is located within the RNA and PB2 binding domains, while the NP mutations I133L and I373T are located within the PB2 and NP binding domains (38). Moreover, the NP amino acid position 100 is located within a surface-exposed cluster of multiple amino acids that confer resistance to the IFN-induced human MxA GTPase (39). Interestingly, the virulence-increasing NP 100I position is also present in the pandemic strains of 1918, 1957, and 1968, in contrast to avian influenza viruses, which contain NP 100R (23, 39). It would be interesting to address whether mutations in HH15-NP would affect MxA restriction.

### TABLE 1 Prevalence of HH05- and HH15-specific mutations among 2009 pH1N1 human isolates

<table>
<thead>
<tr>
<th>Gene product</th>
<th>HH05- or HH15-specific amino acid position</th>
<th>% during pandemic phase (mo/day/yr):</th>
<th>Early (1/1/09–5/15/09)</th>
<th>Mid (5/16/09–6/14/09)</th>
<th>Late (6/15/09–8/09/10)</th>
<th>Post (8/10/10–5/20/2014)</th>
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<td>PB2</td>
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*The analyzed data include amino acid sequences of the protein-encoding genes for all human pH1N1 strains available from the GISAID database from 1/1/09 to 5/20/14.*

*Only positions that differ between HH05 and HH15 were evaluated. HH15-specific amino acid residues are shown in boldface.*

*Time intervals of pandemic phases are defined according to previous classifications (16, 23, 28). Only the frequencies of the polymorphisms specific for HH05 and HH15 are listed, and therefore, the numbers may not add up to 100%.

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**FIG 6** Virus lung titers in recombinant 2009 pH1N1 virus-infected C57BL/6J mice. C57BL/6J mice were either intranasally treated with PBS as a control or infected with 10⁵ PFU of 2009 pH1N1 recombinant virus HH05rec or HH15rec, SGR virus HH05-HAHH15 or HH05-NPHH15, or SPM virus HH05-NP100I, HH05-NPI133L, or HH05-NPI373T. On days 3 and 6 p.i., three animals per time point were sacrificed, lungs were removed, and virus titers were determined by plaque assay. Bars represent means of individual analyses and standard deviations.
and thus provide the later-phase strain with an additional selective advantage in humans.

Remarkably, HH15-specific sites in NP 100I, NA 106I, NS1 123V, and HA 202T were highly prevalent in 2009 pH1N1 strains isolated during the 3rd and most severe wave (2010 to 2011) of the pandemic in terms of hospitalizations and deaths among adults compared to the first two waves in the United Kingdom (40). Further, most HH05-specific sites have almost disappeared among circulating strains in the postpandemic phase. Thus, the virulence-increasing mutations identified in this study might have been under selective pressure and might have affected disease outcome in humans.

In summary, our findings show that viral mutations that have accumulated during pandemic circulation among humans are associated with severe disease outcome in mice. Their high prevalence in currently circulating influenza virus strains further underlines their potential selective advantage in the human population. Furthermore, reassortment of 2009 pH1N1 strains containing intrinsic virulence markers with novel virus subtypes may give rise to the emergence of novel strains of particular virulence and therefore call for high vigilance.

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REFERENCES


