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# Multiple Natural Substitutions in Avian Influenza A Virus PB2 Facilitate Efficient Replication in Human Cells

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**ABSTRACT**

**A strong restriction of the avian influenza A virus polymerase in mammalian cells generally limits viral host-range switching. Although substitutions like E627K in the PB2 polymerase subunit can facilitate polymerase activity to allow replication in mammals, many human H5N1 and H7N9 viruses lack this adaptive substitution. Here, several previously unknown, naturally occurring, adaptive substitutions in PB2 were identified by bioinformatics, and their enhancing activity was verified using *in vitro* assays. Adaptive substitutions enhanced polymerase activity and virus replication in mammalian cells for avian H5N1 and H7N9 viruses but not for a partially human-adapted H5N1 virus. Adaptive substitutions toward basic amino acids were frequent and were mostly clustered in a putative RNA exit channel in a polymerase crystal structure. Phylogenetic analysis demonstrated divergent dependency of influenza viruses on adaptive substitutions. The novel adaptive substitutions found in this study increase basic understanding of influenza virus host adaptation and will help in surveillance efforts.**

**IMPORTANCE**

**Influenza viruses from birds jump the species barrier into humans relatively frequently. Such influenza virus zoonoses may pose public health risks if the virus adapts to humans and becomes a pandemic threat. Relatively few amino acid substitutions—most notably in the receptor binding site of hemagglutinin and at positions 591 and 627 in the polymerase protein PB2—have been identified in pandemic influenza virus strains as determinants of host adaptation, to facilitate efficient virus replication and transmission in humans. Here, we show that substantial numbers of amino acid substitutions are functionally compensating for the lack of the above-mentioned mutations in PB2 and could facilitate influenza virus emergence in humans.**

Influenza A viruses are major human and animal pathogens. In humans, influenza virus infection leads to febrile respiratory illness. Damage to the respiratory epithelium can facilitate infection with other pathogens, resulting in, e.g., secondary bacterial infections (1). Incomplete protection by vaccines and the emergence of viruses resistant to currently used antiviral drugs pose a challenge in the battle against influenza virus infections.

Recurring epidemics and sporadic pandemics account for significant morbidity and mortality among humans, as exemplified by the 1918 pandemic with up to 50 million deaths worldwide (2). Influenza pandemics have been recorded since the Middle Ages (3), with the 2009 influenza pandemic as the most recent incident of a zoonotic virus emerging in an immunologically naive population and spreading worldwide (4). Recent zoonotic infections posing a significant public health threat have occurred with influenza virus subtypes H5N1 and H7N9 (449 and 286 deaths out of 846 and 772 laboratory-confirmed cases, respectively, as reported to WHO until February 2016 [http://www.who.int]). In addition, a number of virus subtypes have been detected in humans more sporadically, such as H6N1, H10N8, and H5N6 (5–7). With the continuous circulation in poultry and repeated transmission to humans, these viruses show a tremendous number of independent adaptation opportunities to cross the species barrier to humans.

Besides amino acid changes in hemagglutinin, nonstructural proteins, and the nucleoprotein, substitutions in the viral polymerase proteins, particularly in the PB2 subunit, have been shown to be major determinants of virulence and adaptation (8–12). Whereas avian influenza A viruses almost exclusively contain glutamic acid at position 627 in the polymerase PB2 protein (PB2-E627), this position is frequently mutated to lysine in human-derived viruses (PB2-E627K) (13), including in the above-mentioned H5N1 and H7N9 viruses (14, 15). The PB2-E627K substitution has been shown to be indispensable for airborne transmission of an H5N1 virus between mammals, presumably by an increase of viral polymerase activity (16). This is further accompanied by improved viral replication at low temperatures and enhanced binding of the viral polymerase to the viral nucleoprotein and importin-α isoforms in human cells (17–19). Intriguingly, the 2009 H1N1 pandemic virus harbors PB2-E627 but possesses polymorphisms at positions 590/591 (PB2-G590S/Q591R) and 271 (PB2-T271A), which were able to partially compensate for the lack of the PB2-E627K substitution (20, 21). Another substitution in PB2, known to have a role in host adaptation, is the exchange of aspartic acid with asparagine at position 701 (PB2-D701N). Several other sub-

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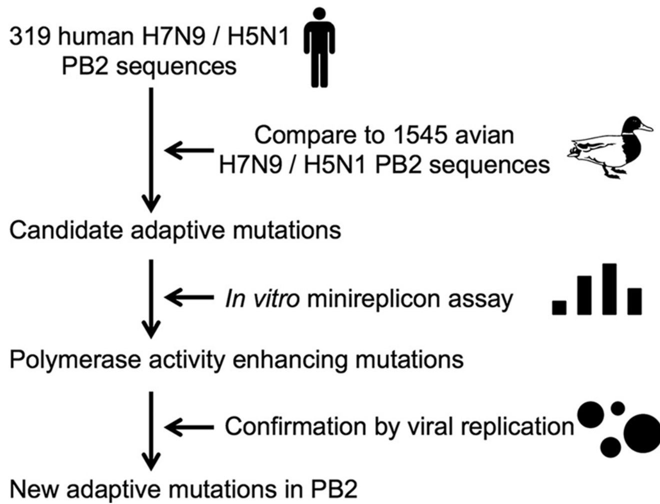


**FIG 1** Scheme for identification of adaptive amino acid substitutions in PB2. Full-length PB2 protein sequences from avian, environmental, and human H5N1 and H7N9 viruses were downloaded from the GISAID EpiFlu database, aligned with MAFFT, and analyzed with UGENE. Candidate substitutions comprising amino acid changes occurring solely or predominantly in human viruses were tested experimentally.

stitutions in PB2, PB1, PA, NP, and NEP have been suggested to enhance polymerase activity in mammalian cells (22–25). However, productive—and sometimes fatal—viral infections of humans with avian viruses of the H5N1 and H7N9 subtypes lacking any previously identified adaptive substitutions have been frequently reported (26).

Here, several new markers for mammalian adaptation of avian influenza A viruses are described in the polymerase subunit PB2. These adaptive amino acid substitutions clustered in the influenza A virus polymerase structure. Phylogenetic analyses indicated variable dependencies on adaptive substitutions of H5N1 and H7N9 viruses.

## MATERIALS AND METHODS

**Biosafety considerations.** The present work was conducted to provide new insights in determinants of influenza A virus host switching and polymerase activity and in support of influenza virus surveillance studies. The work was conducted in agreement with international biosafety and biosecurity regulations, including U.S. Government policies on gain-of-function and dual-use research of concern (http://osp.od.nih.gov/office-biotechnology-activities/biosecurity/dual-use-research-concern). The Biosafety Office of Erasmus Medical Center (MC) ap-

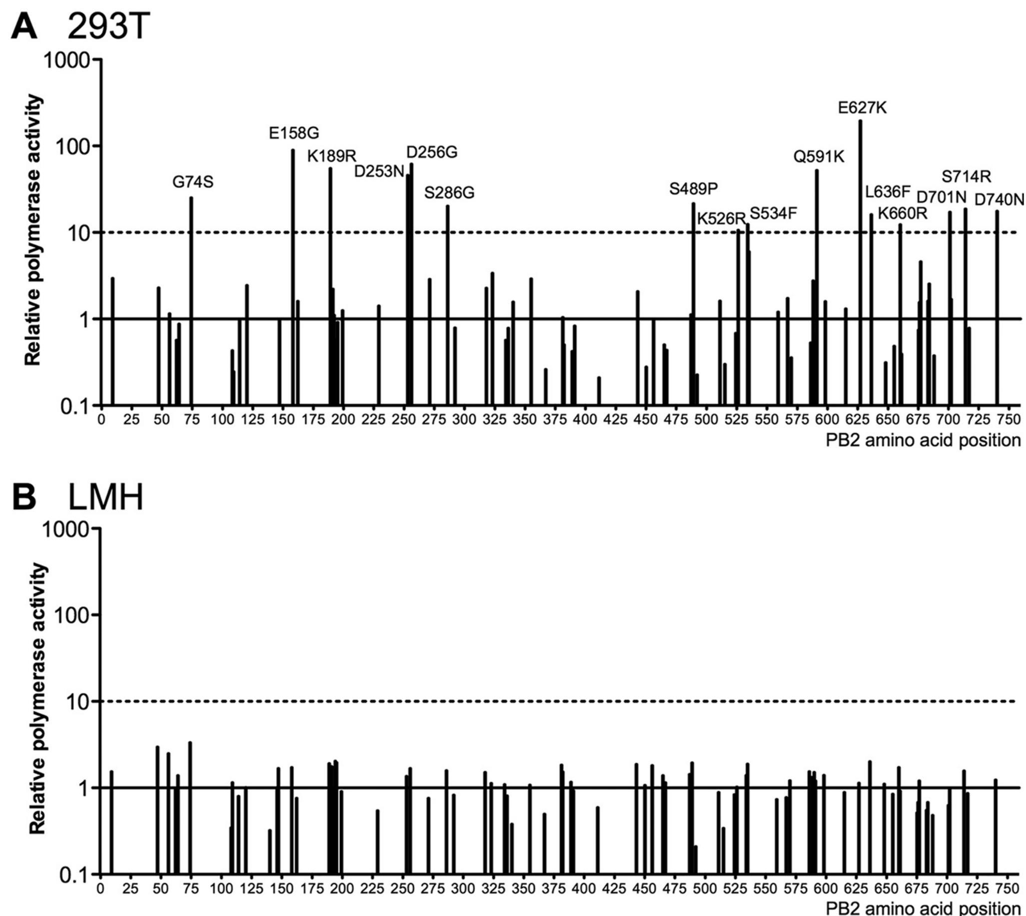


**FIG 2** Avian H5N1 polymerase activity in human and avian cells at 37°C. A polymerase reconstitution assay was performed in human 293T (A) and avian LMH (B) cells at 37°C to screen for adaptive substitutions in the polymerase subunit PB2. Plasmids encoding the avian viral polymerase complex proteins (PB2, PB1, and PA) and the nucleoprotein (NP) were cotransfected with a model vRNA consisting of the firefly luciferase open reading frame flanked by the noncoding regions of segment 8 of influenza A virus. The luminescence of the firefly luciferase reporter was standardized using a plasmid constitutively expressing *Renilla* luciferase protein. The activity of A/chicken/Thailand/ST-351/2008 (H5N1) was set to 1. Positions of amino acid changes that induced at least 10-fold increases in the minireplicon assay are indicated. The 759 amino acid positions of PB2 are shown on the *x* axis.

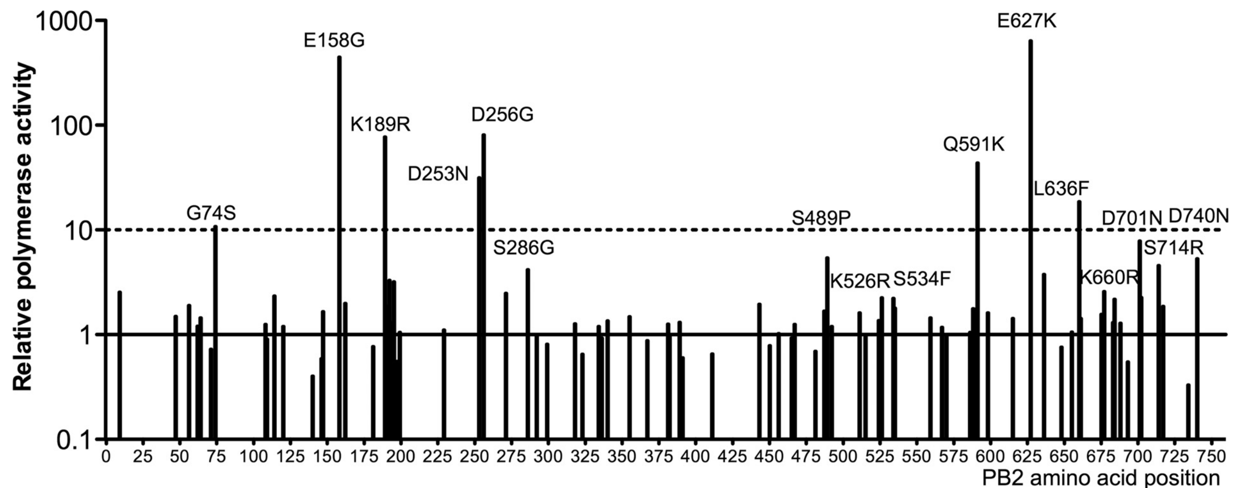


**FIG 3** Avian H5N1 polymerase activity in human cells at 33°C. A polymerase reconstitution assay was performed in human 293T cells at 33°C. Plasmids encoding the avian viral polymerase complex proteins (PB2, PB1, and PA) and the nucleoprotein (NP) were cotransfected with a model vRNA consisting of the firefly luciferase open reading frame flanked by the noncoding regions of segment 8 of influenza A virus. The luminescence of the firefly luciferase reporter was standardized using a plasmid constitutively expressing *Renilla* luciferase protein. The activity of the avian H5N1 A/chicken/Thailand/ST-351/2008 (H5N1) was set to 1. The 759 amino acid positions of PB2 are indicated on the *x* axis.

proved the experiments upon receipt of a permit from the Dutch Government for work with the genetically modified organisms. The work was completed before the U.S. Government Policy for Institutional Oversight of Life Sciences Dual Use Research of Concern came into effect in September 2015. The initial screens to investigate the effect of mutations in the PB2 genes on polymerase activity were conducted using minigenome assays that do not yield replication-competent viruses, under biosafety level 2 (BSL2) conditions. We exclusively tested mutations that were found to occur naturally. Recombinant replication-competent viruses were rescued under BSL3 conditions for confirmation. This was done only after *in vitro* data indicated that they had lower activity than the wild-type viruses, and all mutations could therefore reasonably be anticipated to represent loss of function. For the A/H7N9 virus, we mutated K627 in PB2 to E627 to ensure loss of function and allow evaluation of (partial) functional compensation by other mutations. The experimental work with A/Indonesia/5/2005 (H5N1) mutant viruses was completed prior to the U.S. Government gain-of-function deliberative process and funding pause. Work with A/Anhui/1/2013 (H7N9) mutant viruses was completed upon approval of our conclusion that such experiments did not count as gain-of-function experiments. In accordance with the U.S. Government Policy for Institutional Oversight of Life Sciences Dual Use Research of Concern, the Institutional Oversight of Life Sciences Dual Use Research of Concern Committee of the Icahn School of Medicine at Mount Sinai evaluated our manuscript draft and made recommendations for improvement. As none of the mutations described here enhance influenza virus replication, beyond well-known mutations such as E627K in PB2, we do not consider this publication to describe dual-use research of concern.

**Bioinformatic analysis.** Full-length PB2 protein sequences from avian, environmental, and human H5N1 and H7N9 viruses were downloaded from the GISAID EpiFlu database, aligned with MAFFT, and analyzed with UGENE (27). Candidate substitutions were defined as (i) amino acid variations that were observed at least 4 times in the data set and when the percentage of occurrence in human virus isolates was more than twice that in avian virus isolates or (ii) any substitutions that were observed in human virus isolates at positions where avian virus isolates were 100% conserved.

**Cells.** 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and nonessential amino acids. Chicken hepatocellular carcinoma (LMH) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza) supplemented with 8% FCS, 2% chicken serum, 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and nonessential amino acids. Madin-Darby canine kidney (MDCK) cells were cultured in Eagle's minimal essential medium (EMEM; Lonza) supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodium bicarbonate (Lonza), 10 mM HEPES (Lonza), and nonessential amino acids (MP Biomedicals Europe, Illkirch, France). All cell lines are available from the American Type Culture Collection (ATCC).

**Polymerase reconstitution (minigenome) assay.** A model viral RNA (vRNA), consisting of the firefly luciferase (FF) open reading frame (ORF) flanked by the noncoding regions of segment 8 of an H5N1 influenza A virus (AGCAAAAGCAGGGTGACAAAAACATA-FF-TGATAAAAAC ACCCTTGTTTCTACT) under the control of a human or avian pPolI, respectively, was used for minigenome assays (28). 293T or LMH cells were seeded into 12-well plates, and 25 ng of the reporter plasmid was transfected using Lipofectamine 2000 (Thermo Fisher) along with 50 ng of each of the pCAGGS plasmids encoding PB2, PB1, and PA and 200 ng of the pCAGGS plasmid encoding NP of A/chicken/Thailand/ST-351/2008. Twenty-five nanograms of the *Renilla* luciferase expression plasmid pRL (Promega, Leiden, The Netherlands) served as an internal control to normalize variation in transfection efficiency and sample processing. At 24 h after transfection, luminescence was measured using a Dual-Glo luciferase assay system (Promega) according to the instructions of the manufacturer with a Tecan Infinite F200 machine (Tecan Benelux bv, Giessen, The Netherlands).

**Recombinant viruses.** Reverse genetics plasmids of A/Indonesia/5/2005 (H5N1) and A/Anhui/1/2013 (H7N9) and respective mutant virus plasmids were transfected into 293T cells using Lipofectamine 2000 as described previously (29). Recombinant viruses were propagated in MDCK cells.

**Plaque assays.** MDCK cells were seeded in 6-well plates and at confluence inoculated with virus and grown with a 1.2% Avicel (FMC Biopolymers, Brussels, Belgium) overlay. For comparative analyses, H5N1 virus incubation times were 30 h at 37°C and 40 h at 33°C, while for the H7N9 virus, 48 h at 37°C and 66 h at 33°C were used. After incubation, cells were fixed, washed, permeabilized, and stained with anti-NP monoclonal antibody (1 mg/ml HB65; ATCC) and goat anti-mouse–fluorescein isothiocyanate (FITC) (Invitrogen). Digital images were taken using a Typhoon

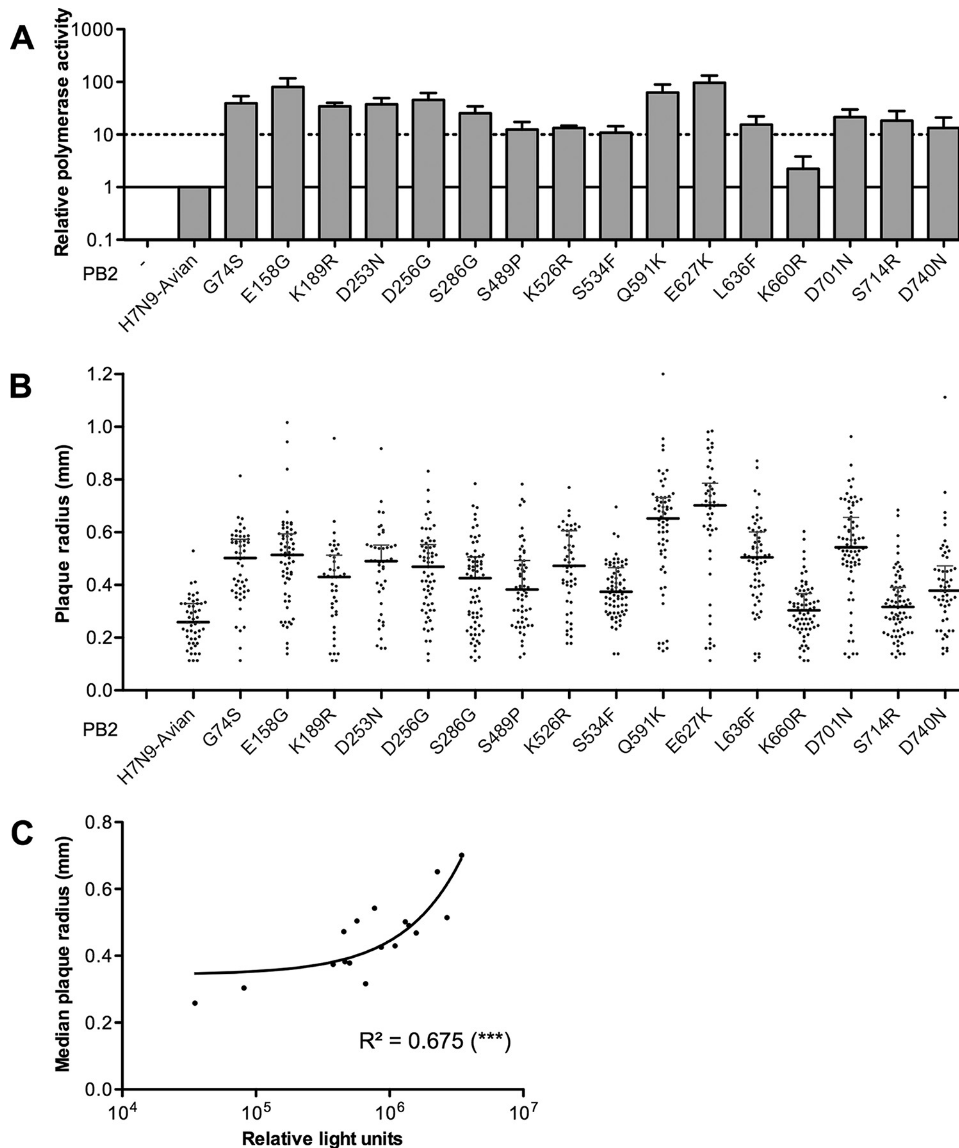


**FIG 4** Avian H7N9 polymerase activity and plaque assays at 37°C. (A) Fold activity of the indicated H7N9 virus mutants was determined as described for Fig. 2A. Avian H7N9 activity was set to 1. (B) One 6-well plate was inoculated with approximately 10 PFU per well of the indicated virus. Plaque size is shown as the median radius with interquartile range. (C) Linear regression was used to describe the trend in median plaque radius in comparison with the respective relative light units of the polymerase determined in the minigenome assay.

system, and plaque size was measured using Image Quant TL software (30) (GE Healthcare Life Sciences).

**Molecular modeling.** The structural model of A/little yellow-shouldered bat/Guatemala/060/2010 (H17N10) (PDB code 4WSB) was used to assign amino acid positions in the influenza A virus polymerase model with the PyMOL Molecular Graphics System, version 1.7.4 (Schrödinger, LLC).

**Phylogenetic analysis.** The set of sequences was aligned using MAFFT. Unrooted phylogenetic trees for each subtype were inferred with PhyML version 3.0 (31), using the general time-reversible (GTR) nucleotide substitution model with a proportion of invariant sites and a gamma distribution of among-site rate variation, all estimated from the data by ModelTest. The trees were visualized using MEGA6 (32).

## RESULTS

**Bioinformatic screening for adaptive substitutions in PB2.** To identify previously unknown, naturally occurring, adaptive changes relevant for influenza A viruses infecting humans, we first performed a bioinformatic screening to identify candidate substitutions involved in the switch from the avian to the human host (Fig. 1). In recent years, viruses of the H5N1 and H7N9 subtypes were responsible for the largest numbers of direct avian-to-human transmission events and therefore represent a multitude of independent adaptation events from birds to humans. We downloaded full-length H5N1 and H7N9 PB2 open reading frame (ORF) sequences of viruses isolated in Asia from humans ($n$ = 319) and birds ($n$ = 1,545) from the GISAID EpiFlu database. Polymorphisms were identified at 239 out of 759 amino acid positions in sequences of human virus isolates (see Table S1 in the supplemental material). In total, 315 amino acid changes were observed from the avian consensus sequence and might be responsible for increased polymerase activity in mammalian hosts.

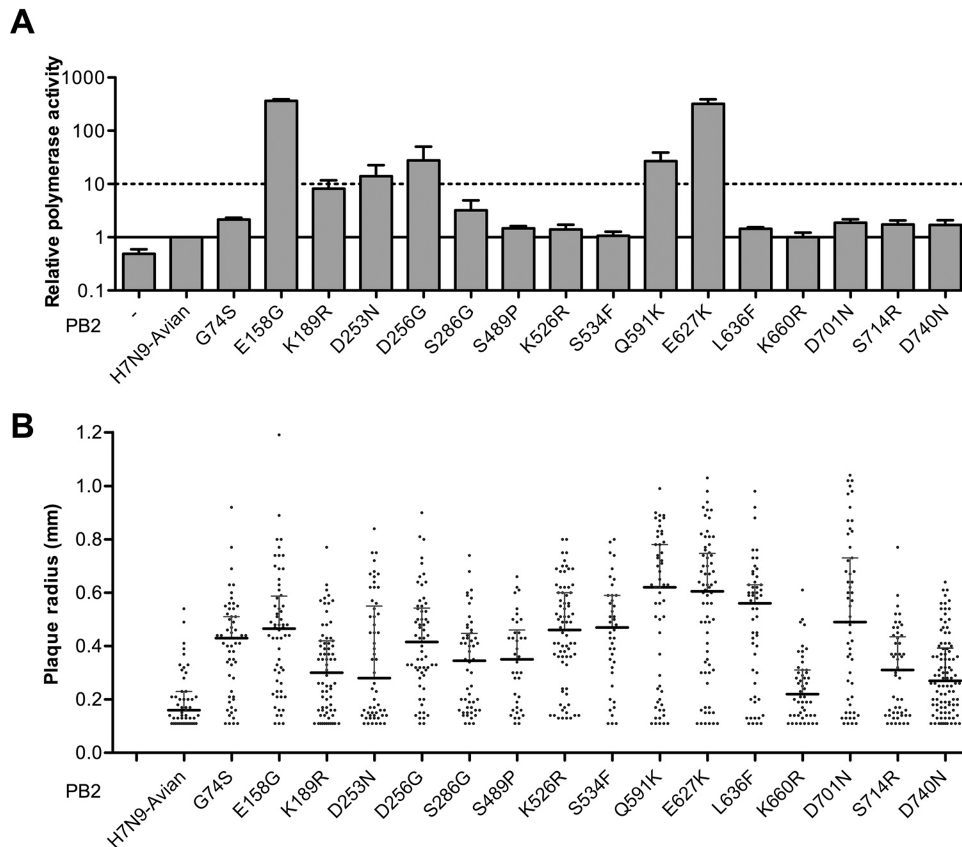


**FIG 5** Avian H7N9 polymerase activity and plaque assay at 33°C. (A) Fold activity of the indicated mutants was determined as described for Fig. 2A, incubating the cells at a temperature of 33°C. H7N9 avian virus activity was set to 1. (B) One 6-well plate was inoculated with approximately 10 PFU per well of the indicated virus. Cells were incubated at 33°C. Plaque size is shown as the median radius with interquartile range.

To screen for such potential beneficial substitutions, two criteria were defined to increase the likelihood of identification of relevant adaptive substitutions that could subsequently be validated experimentally *in vitro*. First, amino acid variations were considered only when they were observed at least 4 times in the data set and the percentage of occurrence in human virus isolates was more than twice that in avian virus isolates. Second, any substitution that was observed in human virus isolates at positions where avian virus isolates were 100% conserved was also selected.

Out of the 315 amino acid substitutions in human virus isolates, 85 fulfilled these criteria. As a control, 9 substitutions known to increase polymerase activity from published studies were also included (PB2-9N, -158G, -256G, -271A, -535T, -588I, -636F, -702R, and -714R) (21, 33–38).

**Several identified substitutions result in increased polymerase activity in an avian H5N1 virus *in vitro*.** To identify substitutions that indeed resulted in increased polymerase activity in human cells, an *in vitro* polymerase reconstitution assay was performed using a firefly luciferase minigenome reporter. Expression plasmids of A/chicken/Thailand/ST-351/2008 (H5N1) served as a prototypic representative for the activity of an avian influenza virus, with its protein sequences of PB2, PB1, PA, and NP showing 20, 52, 38, and 230 identical sequences, respectively, in BLAST searches. In human 293T cells, amino acid substitutions at 16 positions resulted in strongly increased polymerase activity (at least 10-fold), while 9 substitutions moderately increased activity (2.5- to 10-fold) (Fig. 2A; also see Table S2 in the supplemental material). In avian cells, A/chicken/Thailand/ST-351/2008 demonstrated high activity as expected, with amino acid substitutions increasing this activity moderately to a maximum of 3-fold (Fig. 2B). At 33°C (the temperature of the human upper respiratory tract) in human cells, a similar trend as at 37°C, with even more pronounced differences for some mutations, was seen for the polymerase reconstitution assay (Fig. 3). Substitutions that strongly increased polymerase activity were scattered over the entire protein, roughly equally distributed in the N-terminal and C-terminal halves.

**Polymerase activity and replication characteristics of an avian H7N9 virus.** Since the bioinformatics screening was performed for H5N1 as well as H7N9 viruses, we were interested if the substitutions identified as increasing polymerase activity in avian H5N1 virus increased activity in an H7N9 virus background to a similar extent. We used the human virus isolate A/Anhui/1/2013 (H7N9) and mutated PB2 at position 627 to a glutamate, to simulate an avian H7N9 virus (A/Anhui/1/2013-PB2-K627E, exhibiting an amino acid sequence identical to that of the avian virus isolate A/pigeon/Shanghai/S1421/2013).

Amino acid substitutions exhibiting a >10-fold activity increase in the avian H5N1 virus background (Fig. 2A) were introduced into the expression plasmid of A/Anhui/1/2013-PB2-K627E. All 16 amino acid substitutions increased activity to a similar extent as that seen for avian H5N1 virus, with the excep-

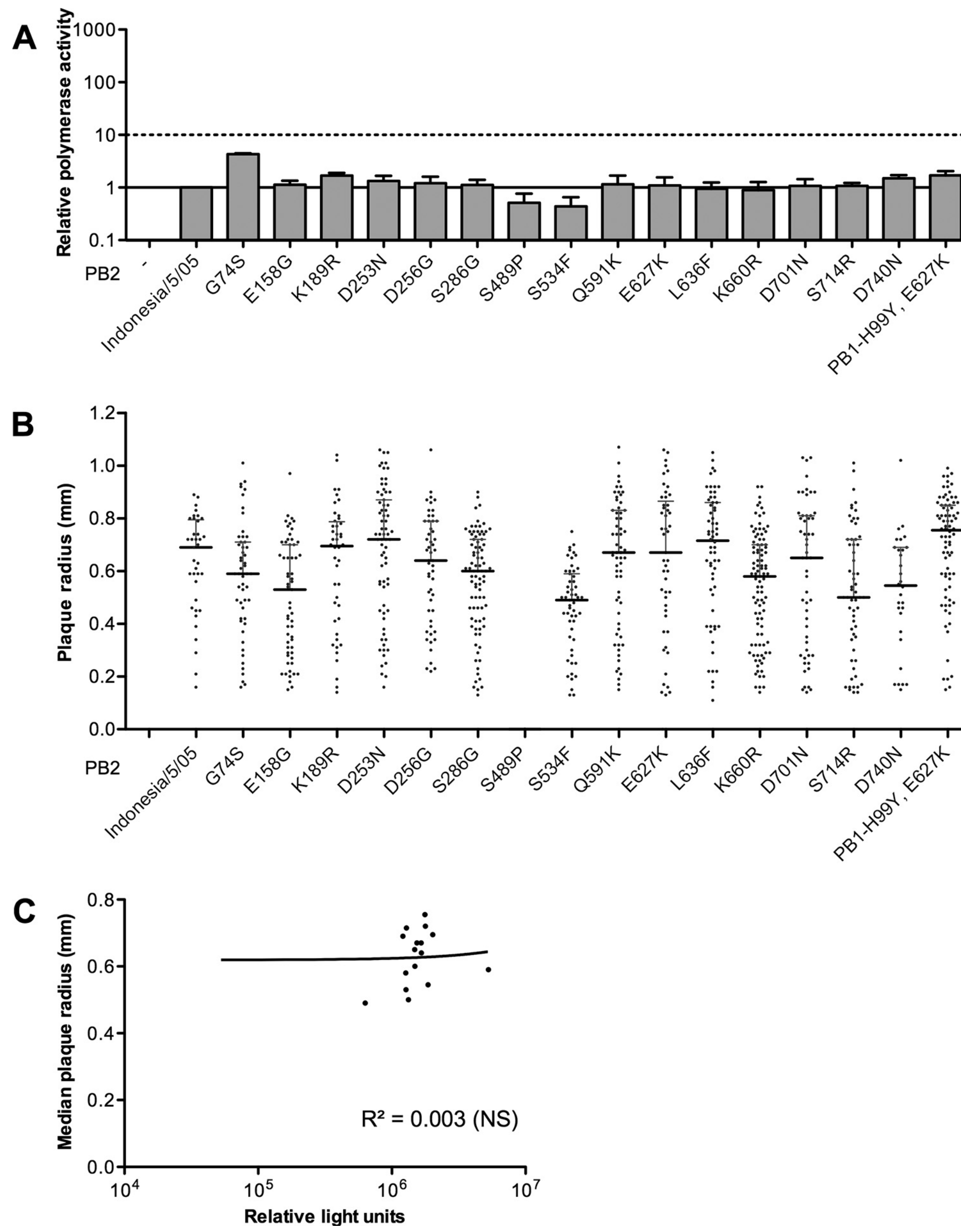


**FIG 6** Human H5N1 polymerase activity and plaque assays at 37°C. (A) Fold activity of the indicated mutants in A/Indonesia/5/05 was determined as described for Fig. 2A, with human A/Indonesia/5/05 activity set to 1. (B) One 6-well plate was inoculated with approximately 10 PFU per well of the indicated virus. Plaque size is shown as the median radius with interquartile range. (C) Linear regression was used to describe the trend in median plaque radius in comparison with the respective relative light units of the polymerase determined in the minigenome assay.

tion of PB2-K660R, which caused only a moderately increased polymerase activity at 37°C (Fig. 4A) and no increase at 33°C (Fig. 5A). We next rescued recombinant viruses, each possessing one of the 16 substitutions in the background of A/Anhui/1/2013-PB2-K627E. A plaque assay on MDCK cells was performed to measure replication of these viruses in mammalian cells (30) (Fig. 4B and 5B). All 16 substitutions significantly increased the plaque radius at 37°C ($P < 0.05$ for PB2-K660R, $P < 0.01$ for PB2-S714R, and $P < 0.001$ for all other mutants). The polymerase activity correlated with plaque radius ($R^2 = 0.675$, $P < 0.001$) (Fig. 4C).

**Polymerase activity and replication characteristics of a human H5N1 virus.** As the human H5N1 virus isolate A/Indonesia/5/05 was shown to be able to acquire airborne transmissibility between ferrets with PB2-E627K and PB1-H99Y in the polymerase complex (16), we used this virus as a model for H5N1 virus adaptation to mammals. A/Indonesia/5/05 and mutants each carrying one of the 16 substitutions were rescued, except the PB2-K526R mutant, as this substitution is already present in the A/Indonesia/5/05 wild-type virus. In contrast to A/Anhui/1/2013-PB2-K627E, A/Indonesia/5/05 already exhibited high polymerase activity (Fig. 6A and 7A; see also Table S3 in the supplemental material). Only minor differences were observed upon introduction of the 16 amino acid substitutions. Only two of the mutants, A/Indonesia/5/05-PB2-D253N and A/Indonesia/5/05-PB2-L636F, pos-

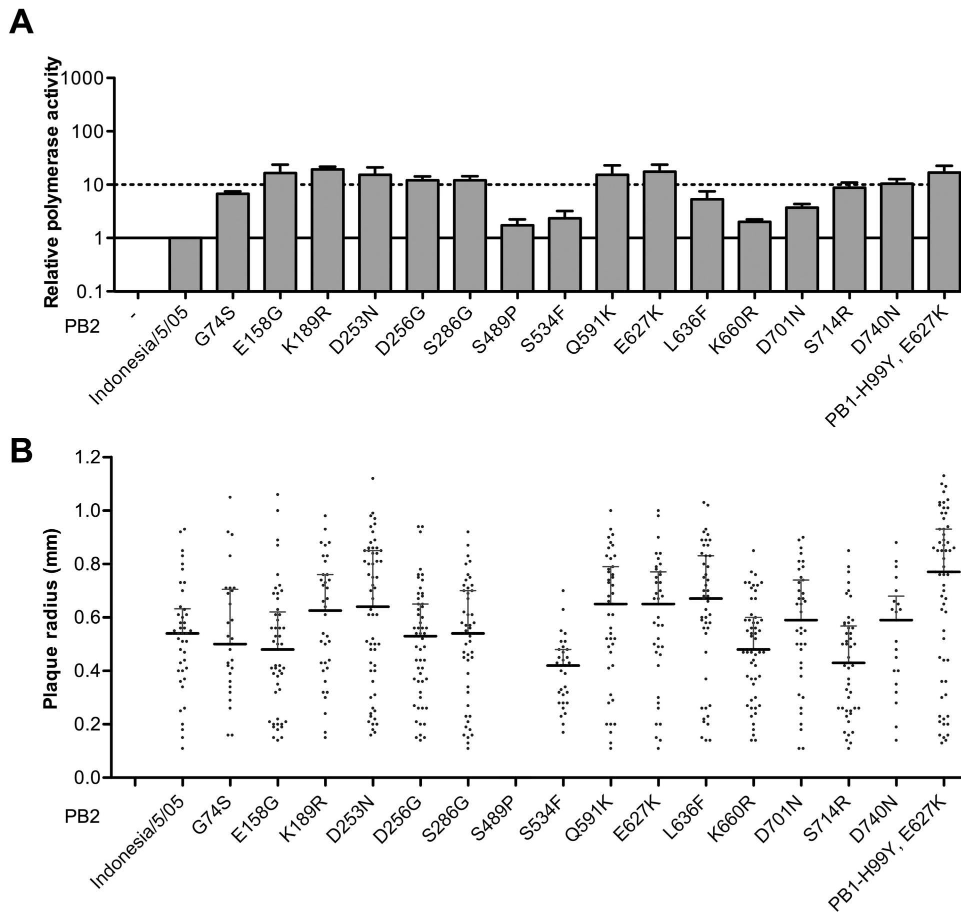


**FIG 7** Human H5N1 polymerase activity and plaque assay at 33°C. (A) Fold activity of the indicated A/Indonesia/5/05 mutants was determined as described for Fig. 2A, incubating the cells at a temperature of 33°C. A/Indonesia/5/05 activity was set to 1. (B) One 6-well plate was inoculated with approximately 10 PFU per well of the indicated virus. Cells were incubated at 33°C. Plaque size is shown as the median radius with interquartile range.

sessed a significantly higher plaque radius than wild-type A/Indonesia/5/05 at 37°C and 33°C (Fig. 6B and 7B) and a plaque radius similar to that of A/Indonesia/5/05-PB1-H99Y-PB2-E627K, specifically at 37°C (Fig. 6B). One mutant (A/Indonesia/5/05-PB2-S489P) could not be rescued. Polymerase activity did not correlate with plaque radius ($R^2 = 0.003$, not significant) (Fig. 6C).

**Structural model of influenza A virus polymerase.** To determine their locations in the polymerase complex and their proximity to each other, the identified substitutions were marked in the recently published structure of the influenza A virus polymerase. Several substitutions surround the well-characterized adaptive position PB2-627 in the 627 domain (591, 636, 714, and 740), while other substitutions were directed toward the inner part of the polymerase complex (158, 253, 256, and 526) (front view) (Fig. 8). Positions 286 and 534 are located between the cap-binding and the 627 domains, while position 74 stands out in being located close to the promoter binding region (back view). Interestingly, only one out of 16 identified positions was not exposed at the protein surface (636).

**Phylogenetic analysis of human virus isolates.** To assess the occurrence of identified adaptive substitutions in PB2 of human H7N9 and H5N1 viruses, we inferred maximum likelihood phylogenetic trees of human virus isolates analyzed in Fig. 1. H7N9 viruses were shown to be strongly dependent on adaptive substitutions, with 66% of the human virus isolates possessing the adaptive substitution PB2-E627K (Table 1) and 88% possessing at least one of the substitutions analyzed in this study (Fig. 9A). A similar picture was seen for H5N1 clade 1 viruses and one cluster of PB2 sequences from clade 2.3 viruses (Fig. 9B). Two other clusters of clade 2.3 viruses showed only a few of the adaptive substitutions described here. Clade 2.1 viruses found in Indonesia revealed polymerase activity-enhancing substitutions already present in avian virus isolates (526R and 660R) (see Table S1 in the supplemental material) with only a few additional adaptive substitutions, such as 286G, 591K, 627K, 740N, and others.

## DISCUSSION

To overcome host restriction of the viral polymerase, influenza A viruses transmitting between humans usually acquired an adaptive substitution(s) in the polymerase subunit PB2. The PB2 lineage that was introduced into the human population around 1918 possesses the marker PB2-627K, while the other lineage, introduced in 2009, contains amino acid substitutions G590S, Q591R, and T271A in PB2 (20, 21). Here, we identified previously uncharacterized substitutions in PB2 (partially) compensating for the lack of these known adaptive changes, in human isolates of H5N1 and H7N9 viruses.

In total, 25 amino acid substitutions were identified, 16

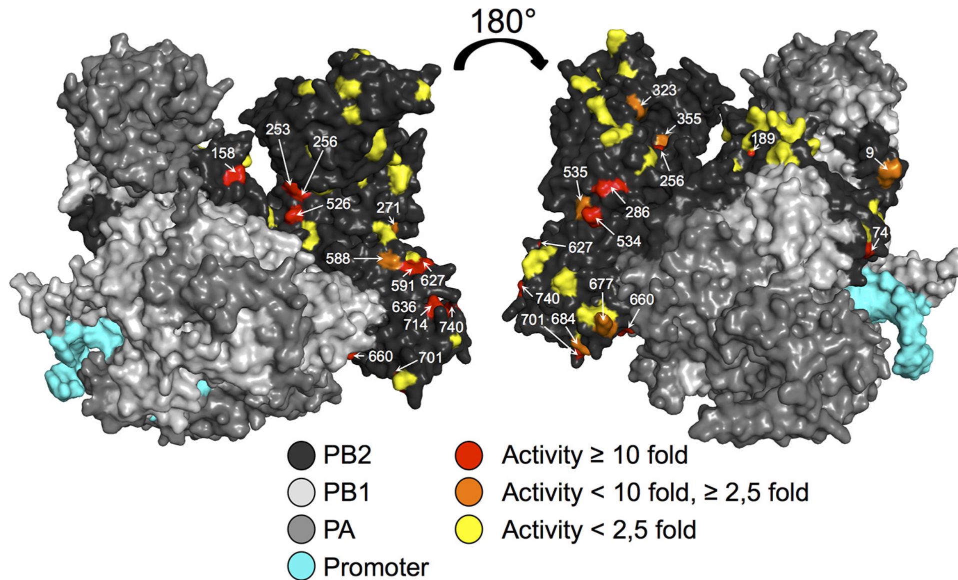


**FIG 8** Localization of adaptive amino acid substitutions in PB2 domains. The program PyMOL was used to assign tested positions from Fig. 2 in the structural model of A/little yellow-shouldered bat/Guatemala/060/2010 (H17N10) (PDB code 4WSB). The three subunits PB2, PB1, and PA are indicated with three shades of gray. Indicated colors were used depending on the fold increase determined in Fig. 2A.

strongly and 9 moderately increasing polymerase activity *in vitro*. All of these were identified in natural human H5N1 and H7N9 viruses. These substitutions were scattered over the entire PB2 protein primary sequence. However, in the central cap-binding domain, only substitutions that moderately increased activity were identified. Recently, the crystal structure of the influenza A virus polymerase has been characterized, providing an excellent opportunity to assign functional domains to the identified substitutions (39). The majority of the identified amino acid changes were located in a putative RNA-exit channel, were surface exposed, and tended toward more basic substitutions, except L636F, which was buried in the structure. Generally, basic substitutions are responsible for RNA binding in RNA binding proteins. As several of the amino acid positions described here are unlikely to be exposed to cellular factors during viral replication, it is tempting to speculate that these modulate RNA binding and thereby alleviate the restriction of the avian influenza A virus polymerase in mammals. Basic substitutions are likely to increase RNA binding and thereby could counterintuitively slow down transcription for enhanced interaction with, e.g., a cellular factor, the viral nucleoprotein, or a second viral polymerase. However, more research is necessary to understand the mechanisms by which these substitutions increase overall polymerase activity. In avian cells, a recently identified potent polymerase-enhancing factor might render the need for further enhancing mutations obsolete (40).

**TABLE 1** Substitutions in human and avian influenza A viruses

| Substitution | % of isolates possessing substitution ($n$) | | | | |
|---|---|---|---|---|---|
| | | Human | | | |
| | Avian (H5N1 + H7N9) (1,397) | H5N1 (231) | H7N9 (116) | pH1N1 (3,750) | H1 to -3 lineage (12,992) |
| S74 | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 |
| G158 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 |
| R189 | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 |
| N253 | 0.0 | 0.0 | 0.9 | 0.1 | 0.0 |
| G256 | 0.0 | 0.0 | 0.9 | 0.1 | 0.1 |
| G286 | 0.5 | 0.9 | 2.6 | 0.2 | 0.0 |
| S489 | 0.0 | 2.2 | 0.0 | 0.0 | 0.0 |
| R526 | 2.0 | 28.6 | 2.6 | 2.7 | 81.4 |
| F534 | 0.1 | 0.0 | 3.4 | 0.0 | 0.0 |
| R/K591 | 0.1 | 3.0 | 5.2 | 99.8 | 1.6 |
| K627 | 7.4 | 24.7 | 65.5 | 0.0 | 98.3 |
| R660 | 2.0 | 5.2 | 0.0 | 6.0 | 0.2 |
| N701 | 0.1 | 6.1 | 9.5 | 0.1 | 0.5 |
| R714 | 0.0 | 0.0 | 0.0 | 0.1 | 0.1 |
| N740 | 1.1 | 3.9 | 0.0 | 0.1 | 1.1 |

During the first half of 2015, there were already more than 300 human cases of infection with H5N1 and H7N9 viruses (http://www.who.int), demonstrating the topicality of both subtypes. Although the minireplicon system used in this study has its limitations, as it does not contain all polymerase activity-influencing factors, e.g., NEP (10, 41) and NS1 (42), and not all 8 segments are expressed, it is broadly used to investigate activity changes and fundamental mechanisms. Here, we confirmed functionality for 14 out of 16 ($P < 0.001$) amino acid substitutions in an avian H7N9 virus background. Of these, several substitutions (74S, 189R, 286G, 489P, 534F, and 660R) have not been characterized before, or only recently (740N) (43). We observed a good correlation between polymerase activity and median plaque radius, suggesting that the minireplicon assay indeed is a good screening assay for human adaptive substitutions in the polymerase complex of avian influenza viruses. The plaque radius was highest for substitutions most frequently occurring in human virus isolates. Animal models could be used to further investigate how the identified substitutions increase fitness. Pathogenicity in mice can be assessed to study the potential virulence in mammals, while experiments with ferrets would help to determine whether the identi-

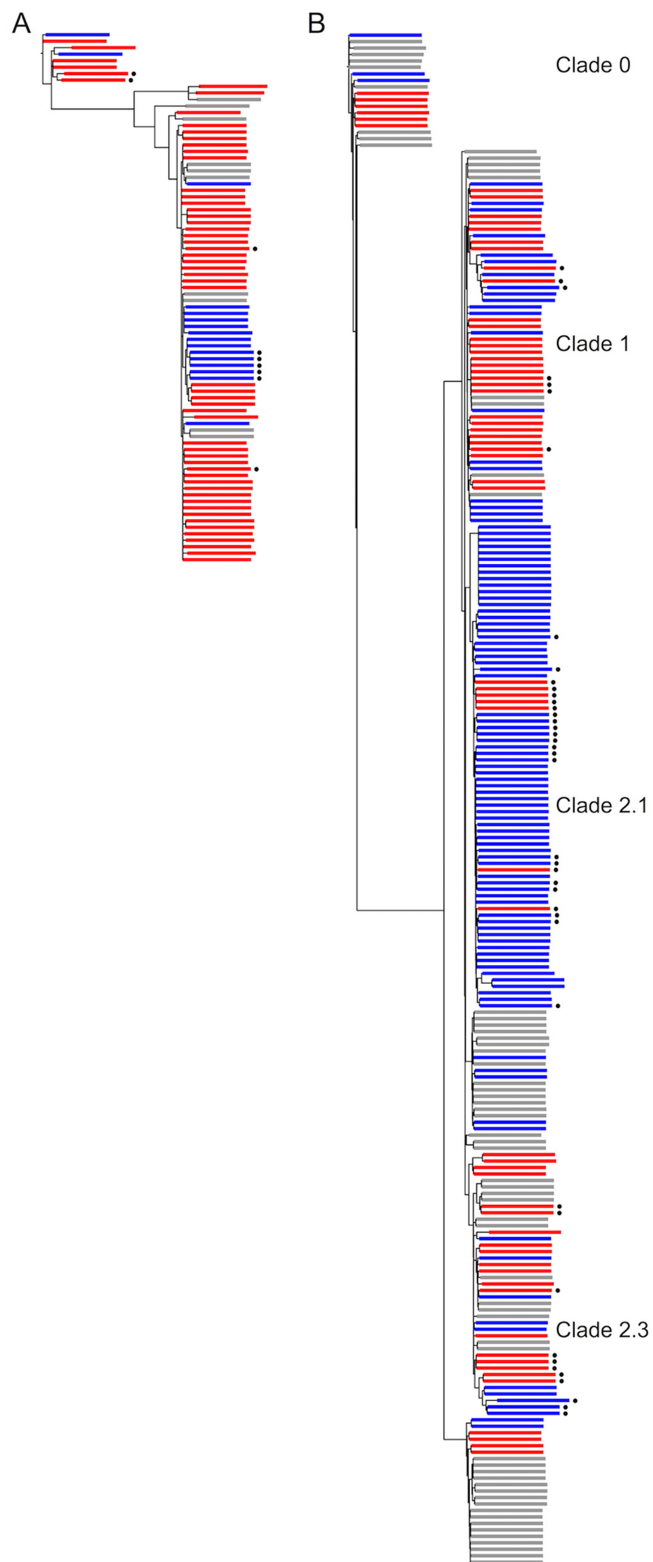


**FIG 9** Phylogenetic PB2 trees. Maximum likelihood phylogenetic trees of human H7N9 (A) and H5N1 (B) PB2 sequences were estimated under the general time-reversible model using PhyML. Virus isolates highlighted in red possess the adaptive substitution PB2-E627K; isolates in blue possess any other identified adaptive substitution described here and in references 23, 24, and 48. A black dot indicates that there is more than one identified adaptive mutation.

fied substitutions can increase the potential of these viruses to transmit between mammals.

In the phylogenetic tree (Fig. 9), a strong dependency on 627K, but also 591K and 701N, can be seen. Newly identified substitutions, such as S286G and S534F, mainly occur in combination with 627K and 591K and might further boost polymerase activity above the level of the E627K substitution alone. Correspondingly, the human-transmissible H3N2 and pH1N1 viruses have been reported to be dependent on multiple adaptive mutations (21, 44–46). Additionally, adaptation of the polymerase subunits is not limited to polymerase activity but might also help to circumvent the host innate immune response (18, 33).

For the human H5N1 virus isolate A/Indonesia/5/05, no correlation between polymerase activity and median plaque radius was observed as has been shown for the substitution PB2-E627K before (16). In the previous study, a stronger increase of polymerase activity by PB2-E627K has been observed than that reported here, most likely due to the different methods applied. Here, we used lower concentrations of transfected plasmids and a different reporter system. The lack of correlation between polymerase activity and median plaque radius observed here (Fig. 6) for A/Indonesia/5/05 mutants might be due to the high polymerase activity of the wild-type virus likely caused by PB2-526R and PB2-323L and the enhancing activity of NEP-7L (10), or a contribution of unknown factors in PB2, PB1, or PA. The phylogenetic tree revealed that only 9% of the Indonesian clade 2.1 viruses possess 627K, suggesting that there was a low dependency on acquiring this adaptive substitution. An H5N1 virus adapted for airborne transmission between ferrets was shown to be strongly dependent on the PB2-E627K substitution but required PB1-H99Y for fine-tuning of activity (16, 47).

In contrast to clade 2.1, clade 1 H5N1 viruses were strongly dependent on adaptive substitutions in PB2 that are uncommon in the avian reservoir. The same is seen for a group of clade 2.3 viruses; however, 2 other groups hardly showed any adaptive substitutions. This might be due to a stronger dependency on adaptive substitutions in PB1 or PA but also to an already high activity of the polymerase before switching the host species to mammals, omitting the need to further adapt the polymerase activity in humans.

The number of previously known adaptive mutations in addition to the substitutions newly identified here shows that there are many functionally equivalent mutations enabling influenza A viruses to acquire high polymerase activity in mammalian cells. However, human isolates were found to be strongly dependent on basic amino acid substitutions in PB2. Additionally, specific mutations appear to have strain-dependent effects. This opens the possibility to assign potentially beneficial mutations to specific lineages and update surveys such as the CDC H5N1 Genetic Changes Inventory (http://www.cdc.gov/flu/avianflu/h5n1-genetic-changes.htm) with approaches like this one, to efficiently support surveillance studies.

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