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PB2 subunit of avian influenza virus subtype H9N2: a pandemic risk factor

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Avian influenza viruses of subtype H9N2 that are found worldwide are occasionally transmitted to humans and pigs. Furthermore, by co-circulating with other influenza subtypes, they can generate new viruses with the potential to also cause zoonotic infections, as observed in 1997 with H5N1 or more recently with H7N9 and H10N8 viruses. Comparative analysis of the adaptive mutations in polymerases of different viruses indicates that their impact on the phylogenetically related H9N2 and H7N9 polymerases is higher than on the non-related H7N7 and H1N1pdm09 polymerases. Analysis of polymerase reassortants composed of subunits of different viruses demonstrated that the efficient enhancement of polymerase activity by H9N2-PB2 does not depend on PA and PB1. These observations suggest that the PB2 subunit of the H9N2 polymerase has a high adaptive potential and may therefore be an important pandemic risk factor.

INTRODUCTION

Aquatic birds are the natural reservoir from which influenza A viruses are occasionally transmitted to terrestrial birds and mammals. Most of these transmissions are transient and do not result in a stable virus lineage. However, on rare occasions, the virus adapts to the new species and may then cause a pandemic. Mutations responsible for a shift in host specificity have been observed in most viral proteins (Chen *et al.*, 2006; Klenk *et al.*, 2008; Cauldwell *et al.*, 2014), including the polymerase (Gabriel *et al.*, 2013; Gabriel & Fodor, 2014). A particularly important determinant of host range appears to be the PB2 (polymerase basic 2) subunit of the polymerase, as numerous adaptive mutations have been observed in this protein (Subbarao *et al.*, 1993, 1998; Gabriel *et al.*, 2005; Labadie *et al.*, 2007; Bussey *et al.*, 2010; Wang *et al.*, 2012; Czudai-Matwich *et al.*, 2014; Zhang *et al.*, 2014; Li *et al.*, 2015). Amongst these, mutation E627K in the PB2 subunit of the polymerase complex has been known for a long time to play a prominent role in the adaptation of avian viruses of various subtypes to mammalian hosts (Subbarao *et al.*, 1993, 1998; Li *et al.*, 2012; Zhang *et al.*, 2014). Another important determinant of mammalian specificity is the adaptive mutation D701N also observed with different viruses (Gabriel *et al.*, 2005; Li *et al.*, 2005; Steel *et al.*, 2009). Two adaptive mutations located nearby, PB2 S714I and S714R, have been found less frequently (Czudai-Matwich *et al.*, 2014). All of these mutations enhance polymerase activity, virus replication and pathogenicity of an avian virus in a mammalian host (Gabriel & Fodor, 2014).

H9N2 viruses are low-pathogenic avian influenza viruses that have been observed worldwide and are endemic in large parts of Asia (Xu *et al.*, 2007; Sun *et al.*, 2010). They have been isolated not only from birds, but also from pigs (Cong *et al.*, 2007, 2008; Yu *et al.*, 2011) and humans (Peiris *et al.*, 1999; Butt *et al.*, 2005). In 2009, PB2 mutation D701N was detected in a human H9N2 isolate [GenBank accession number KF188313], further supporting the concept that this mutation promotes transmission and adaptation to a mammalian host. H9N2 viruses isolated from birds have also been described to efficiently infect mice with (Park *et al.*, 2015) and without (Choi *et al.*, 2004) prior adaptation. Finally, H9N2 viruses are frequently observed in terrestrial poultry. They reassort with co-circulating strains generating new viruses with the potential to cause zoonotic infection, as was the case with H5N1 virus in 1997 (Guan *et al.*, 1999), H7N9 virus in 2013 (Feng *et al.*, 2013; Gao *et al.*, 2013) and H10N8 virus in 2014 (Chen *et al.*, 2014; Qi *et al.*, 2014). The possibility that H9N2 strains may contribute to the evolution of a new pandemic virus is therefore a matter of considerable concern.

Here, we show that PB2 mutations E627K, D701N and S714R enhance polymerase activity and viral replication in mammalian cells as well as pathogenicity of influenza virus A/quail/Shantou/2061/2000 (H9N2) in mice.

Furthermore, comparison with other influenza A viruses indicates that the effect of these mutations on polymerase activity is most distinct when introduced into the PB2 subunit of H9N2 virus or into H9N2-related PB2. These observations support the concept that H9N2-like PB2 enhances the potential of an avian influenza A virus to adapt to a mammalian host and highlight it as a potential pandemic risk factor.

RESULTS

Adaptive mutations E627K, D701N and S714R in PB2 of H9N2 virus enhance polymerase activity and viral growth in mammalian cells

To investigate the role of PB2 mutations E627K, D701N and S714R, we have selected the H9N2 strain that contains the avian-type signatures at these positions (627E, 701D and 714S). Constructs of H9N2 PB2 containing the mutations alone or in combination were generated by site-directed mutagenesis. Subsequently, the activities of the reconstituted polymerases were analysed in HEK 293T cells in a minigenome assay by quantifying firefly and *Renilla* luciferase production. Activities of mutant polymerases were compared with the activity of the avian polymerase (627E–701D–714S) used as internal standard (Fig. 1). Introduction of mutation E627K resulted in a 20-fold increase, whereas mutations D701N and S714R induced a six- and eightfold increase, respectively. Double mutation E627K–D701N did not alter the polymerase activity compared with E627K alone, but the double mutation E627K–S714R induced a 27-fold increase. Double mutation D701N–S714R led to a slightly higher activity as well when compared with the single-point-mutants D701N or S714R. The highest effect with a 38-fold increase in polymerase activity was observed when the three mutations were combined. These results confirmed the previous observation that mutation E627K enhances the activity of the H9N2 polymerase (Wu *et al.*, 2009; Li *et al.*, 2012; Wang *et al.*, 2012; Sang *et al.*, 2015) and showed that introduction of the other mutations resulted in even further increase.

In order to study the effects of these mutations on virus replication, we generated H9N2 recombinant viruses containing the adaptive mutations E627K, D701N and S714R alone or in combination. Human airway epithelial cells (Calu-3 cells) were infected at m.o.i. 10^{-4}, and supernatants were collected at 10, 24, 48 and 72 h post-infection (p.i.). Viruses containing single mutations E627K, D701N and S714R grew to titres that were 100-, 500- and sevenfold higher, respectively, compared with WT virus at 24 h. There was a 1000-fold increase in titre observed with triple-mutant E627K–D701N–S714R (Fig. 2). The titres obtained upon infection with double-mutants E627K–D701N, E627K–S714R and D701N–S714R were 20- to 200-fold increased compared with WT virus (data not shown). Thus, we showed that all adaptive mutations analysed here increased the replication of H9N2 virus in human cells, and that this effect was more distinct with mutations E627K and D701N than with mutation S714R. Furthermore, there were cooperative effects (see Discussion) when the virus contained combinations of these mutations.

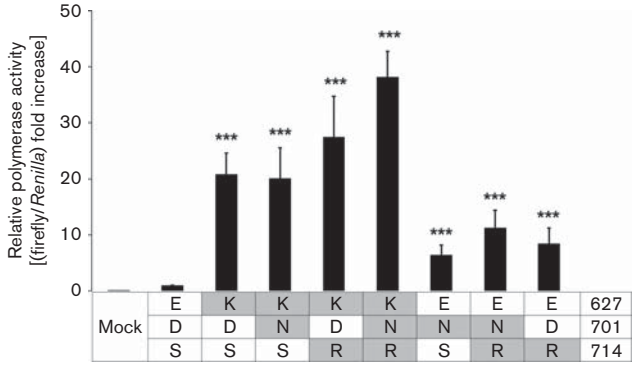

Fig. 1. Influence of PB2 mutations on H9N2 polymerase activity in mammalian cells. HEK 293T cells were transfected with pHW2000 constructs encoding PB1, PA, PB2 and NP (nucleoprotein) of H9N2 virus, together with reporter constructs encoding firefly and *Renilla* luciferase. The amino acid signatures at positions 627, 701 and 714 of PB2 are shown (grey, mammalian; white, avian). Activities are indicated relative to the polymerase complex containing the avian signature 627E–701D–714S in PB2 and set to 1. As background control, plasmids were transfected omitting the PB2 subunit (Mock). Data represent mean ± SD of at least three independent experiments. ***$P<0.001$.

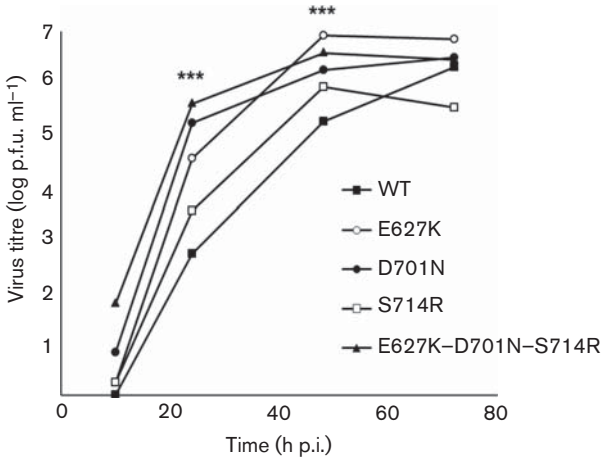

Fig. 2. Enhancement of virus growth in mammalian cells. Calu-3 cells were infected with H9N2-WT virus or virus containing adaptive mutations E627K, D701N and S714R (m.o.i. 10^{-4}). Supernatants were taken at 10, 24, 48 and 72 h p.i. WT represents virus containing PB2 with the avian signature 627E–701D–714S. Virus titration was performed by plaque assay on MDCKII cells. Data represent mean of logarithmic virus titres of three independent experiments. ***$P<0.001$.

PB2 mutations enhance pathogenicity of H9N2 virus in mice

To determine whether the mutations alter the pathogenicity of the virus, we infected mice with the H9N2 recombinant viruses. BALB/c mice were infected with a dose of 10^6 p.f.u. of each virus, and monitored for weight loss and survival (Fig. 3). When compared with mock-infected mice, no weight loss was observed after infection with WT virus or virus containing mutation D701N. Infection with mutant viruses S714R and E627K induced a slight-to-moderate reduction in weight (5 and 10 %, respectively) at 4 days p.i. The most distinct effect with 20 % reduction in weight was observed after infection with the triple-mutant E627K–D701N–S714R (Fig. 3a). The survival rates reflected more or less the weight loss (Fig. 3b). All mice infected with WT, D701N and S714R viruses survived, and did not present any signs of illness. Mice infected with virus containing mutation E627K showed disease symptoms, although no lethality was observed. The most distinct effect with 20 % reduction in weight and 40 % lethality was observed upon infection with the triple-mutant virus.

We then determined virus titres in lung and brain of the infected animals, which correlated, in general, with the observed weight loss and survival rates (Fig. 3c). Lungs of mice infected with WT or S714R viruses presented the lowest viral titres (2.6×10^3 and 4.4×10^4 p.f.u. ml^{-1}, respectively), whereas infection with mutation E627K or the triple mutant resulted in lung titres of 1.1×10^5 and 5.1×10^4 p.f.u. ml^{-1}, respectively. Surprisingly, mice infected with virus containing adaptive mutation D701N showed a comparatively high lung titre (5.6×10^4 p.f.u. ml^{-1}) that did not correlate with the negligible effect of this mutation on body weight loss. No virus titres were detected in the brain of infected animals.

Taken together, these data showed that the adaptive PB2 mutations E627K, D701N and S714R increased the pathogenicity of the H9N2 virus in mice to varying extents and that disease symptoms were most distinct after infection with the triple mutant.

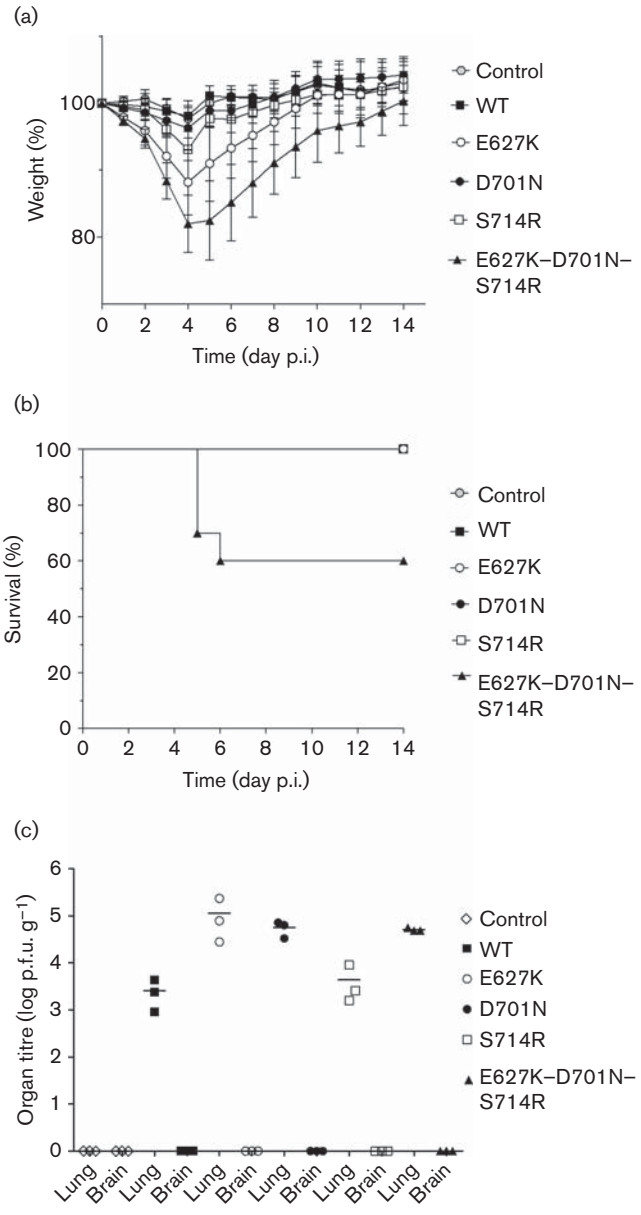

Fig. 3. Effects of PB2 mutations on the pathogenicity of H9N2 virus in mice. Female BALB/c mice were infected intranasally with 10^6 p.f.u. H9N2 WT virus, the single-point-mutant viruses E627K, D701N or S714R or the triple-mutant virus. Control mice received PBS (Control). (a) Weight loss and (b) survival of infected mice (n=5 per group) were measured daily for 2 weeks. (c) At day 3 p.i., tissues were collected (lung and brain; n=3 per group) for determination of viral organ titres. Viral titres [p.f.u. (g organ weight)$^{-1}$] were determined by plaque assay on MDCKII cells. Data represent individual logarithmic virus titres of each organ and their mean (n=3).

With H9N2 and H7N9 viruses, adaptive mutations had a higher impact on polymerase activity than with H1N1pdm09 and H7N7 viruses

We have shown above that introduction of mutations E627K, D701N and S714R into the PB2 subunit of H9N2 virus induced a 20-, six- and eightfold increase of polymerase activity, respectively, in mammalian cells. As these mutations have also been found to mediate host adaptation of other influenza viruses (Gabriel *et al.*, 2005; Czudai-Matwich *et al.*, 2014), it was of interest to find out whether there were strain-specific variations. For this purpose, we analysed polymerases from phylogenetically related and unrelated influenza A viruses. The variations in the PB2 sequences are shown in Table 1.

As non-related viruses, we chose A/Hamburg/05/2009 (H1N1pdm09) (Otte *et al.*, 2011) and the SC35 variant of A/seal/Massachusetts/1/1980 (H7N7) (Li *et al.*, 1990; Gabriel *et al.*, 2005), and analysed the polymerase activity

Table 1. Variations in the PB2 amino acid sequences of the A/quail/Shantou/2061/2000 (H9N2), A/Hamburg/05/2009 (H1N1pdm09), A/seal/Massachusetts/SC35/1980 (H7N7) and A/Anhui/1/2013 (H7N9) viruses.

Position	H9N2	H1N1pdm09	H7N7	H7N9
54	K	**R**	K	K
65	E	**D**	E	E
106	A	**T**	**T**	**T**
125	L	L	**M**	L
147	M	**T**	**I**	**I**
161	E	**D**	**D**	**D**
184	T	**A**	T	T
190	R	**K**	**K**	**K**
192	E	E	**K**	E
194	K	**Q**	**Q**	**Q**
195	N	**D**	**D**	**D**
197	N	**K**	**K**	**K**
225	S	**G**	**I**	S
255	V	V	**I**	V
271	T	**A**	T	T
292	V	V	**I**	V
299	K	**R**	**R**	**R**
315	M	**I**	M	M
318	K	**R**	**R**	**R**
334	K	**S**	**S**	**R**
340	R	**K**	R	R
355	K	**R**	**R**	**R**
381	M	**L**	**L**	**L**
441	N	**D**	**D**	**D**
453	P	**S**	P	P
478	V	**I**	**I**	V
508	Q	**R**	**R**	**R**
524	M	**T**	**T**	**T**
526	K	**R**	K	K
547	V	V	**I**	V
559	T	**I**	T	**N**
567	E	**D**	**D**	**D**
588	A	**T**	A	A
590	S	S	**G**	**G**
591	Q	**R**	Q	Q
645	M	**L**	M	M
649	V	V	**I**	V
655	A	**V**	**V**	**V**
661	T	**A**	**A**	**A**
667	I	**V**	**V**	**V**
684	A	**S**	A	A
717	T	**A**	**A**	**A**

In bold are highlighted the amino acid differences compared to the H9N2-PB2 sequence.

by minigenome assay (Fig. 4). Introduction of mutation E627K induced a threefold increase in H1N1pdm09 and a sevenfold increase in H7N7 polymerase activity, compared with a 20-fold increase in H9N2 polymerase activity as pointed out earlier. Mutation D701N induced a twofold increase in H1N1pdm09 and H7N7 polymerase activity, compared with a fivefold increase in H9N2. Introduction of S714R induced a twofold increase in H1N1pdm09 and H7N7 polymerase activity, compared with a sixfold increase in H9N2. Finally, the combination of E627K and S714R induced a sevenfold increase in H1N1pdm09 and a 11-fold increase in H7N7 polymerase activity, compared with a 27-fold increase in H9N2 polymerase activity. These results demonstrated that introduction of the adaptive mutations had a higher impact on polymerase activity in H9N2 virus than in H1N1pdm09 or H7N7 viruses.

As phylogenetically related virus, we used A/Anhui/1/2013 (H7N9) virus (Feng *et al.*, 2013), containing the internal genes of a H9N2 virus (Feng *et al.*, 2013). As H7N9-PB2 WT already contains the E627K mutation, mutations D701N and S714R as well as revertant K627E were generated for comparative analysis (Fig. 4). The results showed that mutation E627K induced a 15-fold increase, mutation D701N a threefold increase and mutation S714R a twofold increase in H7N9 polymerase activity. Combination of mutation E627K with mutation S714R led to a 25-fold increase in H7N9 polymerase activity. These values were similar to those observed with H9N2 polymerase (20-, six-, eight- and 27-fold, respectively).

These observations indicated that the adaptive mutations increased the activity of all polymerases analysed, but that their impact on the H9N2 and the related H7N9 polymerases was significantly higher than on the non-related H7N7 and H1N1pdm09 polymerases.

Increase in polymerase activity does not depend on PA and PB1

Next, it was of interest to clarify whether the constellation of PB2 with PA (polymerase acidic) and PB1 (polymerase basic 1) subunits affects the increased sensitivity of the H9N2 polymerase to the adaptive mutations. We therefore generated polymerase complexes of H1N1pdm09, H7N7 and H7N9 viruses in which the subunits were replaced individually by their H9N2 analogues and analysed the effect of the adaptive PB2 mutations in these reassortants.

First, we compared the H1N1pdm09 polymerase with H1N1pdm09–H9N2 polymerase reassortants (Fig. 5a). Introduction of H9N2-PA into the H1N1pdm09 polymerase led to a drop of activity of all mutants, whereas introduction of H9N2-PB1 did not affect their activities when compared with the H1N1pdm09 polymerase. However, when H9N2-PB2 was introduced, the stimulating effects of the adaptive mutations on polymerase activity were significantly enhanced and in the range of those observed with H9N2 polymerase (cf. Fig. 4). Likewise, introduction of H9N2-PA and H9N2-PB1 into H7N7 polymerase decreased the activity, whereas in the reassortant containing H9N2-PB2 the effects of the adaptive mutations were quite distinct (Fig. 5b), equalling those observed with the authentic H9N2 polymerase (cf. Fig. 4).

We then analysed the activities of the reassortants of the related H9N2 and H7N9 polymerases (Fig. 5c). Upon

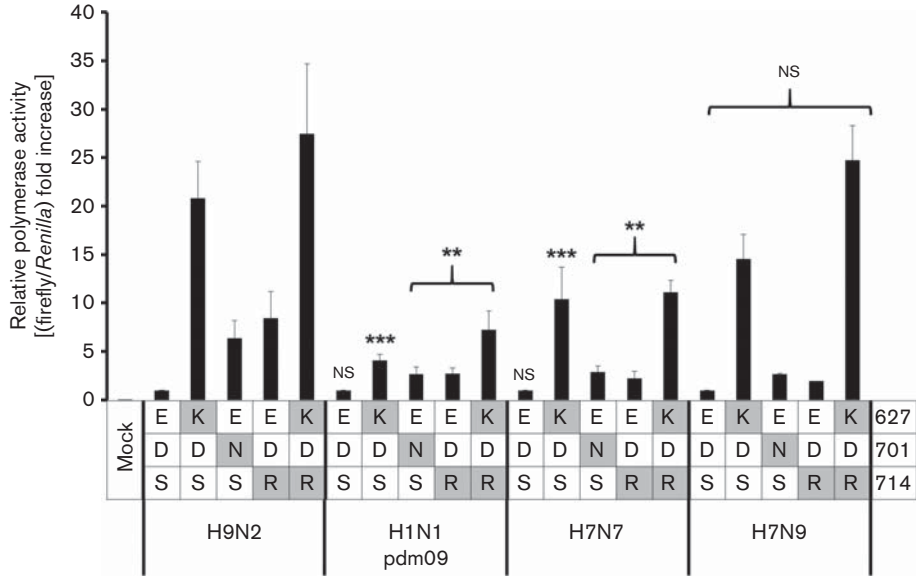

Fig. 4. Impact of PB2 mutations on activities of different viral polymerases. HEK 293T cells were transfected with pHW2000 constructs encoding PB1, PA, PB2 and NP of H9N2, H1N1pdm09, H7N7 and H7N9 viruses. Polymerase activities were determined by dual-luciferase assay. Activities are indicated relative to the polymerase complex containing the avian signature 627E–701D–714S in PB2 and set to 1. As a background control, plasmids were transfected omitting the PB2 subunit (Mock). The amino acid signatures at positions 627, 701 and 714 of PB2 of H9N2, H1N1pdm09, H7N7 and H7N9 polymerases are shown (grey, mammalian; white, avian). Data represent mean ± SD of at least three independent experiments. Statistical significance of the polymerase increase due to avian/mammalian signature was defined as $P<0.05$ (**$P<0.01$; ***$P<0.001$; NS, non-significant) (Student's *t*-test). The mutants of the H1N1pdm09, H7N7 and H7N9 polymerases were compared with the respective H9N2 polymerase mutants.

replacement of H7N9-PB1 and H7N9-PA by the H9N2 subunits, we observed a moderate reduction in polymerase activity. However, there was no complete loss of activity as observed after introduction of H9N2-PA into H1N1pdm09 and H7N7 polymerases. Furthermore, introduction of H9N2-PB2 harbouring host-adaptive mutations did not significantly alter the activity when compared with the homologous H7N9 polymerase. Mutations E627K, D701N and S714R, and the double mutation E627K–S714R, led to a 12-, four-, five- and 31-fold increase, respectively, in polymerase activity of reassortant H7N9/PB2-H9N2 as compared with a 14-, three-, two- and 24-fold increase, respectively, with the authentic H7N9 polymerase.

These observations confirmed that the enhancement of polymerase activity by the adaptive PB2 mutations was most distinct with the H9N2 virus and the related H7N9 virus, and they showed that the enhancing effect was a specific characteristic of H9N2-PB2 without any significant contribution of H9N2-PA and -PB1.

DISCUSSION

Within the last 25 years, H9N2 outbreaks in poultry have been frequently documented throughout Europe, the Middle East and parts of Asia. Occasionally, these viruses have been transmitted to man, and co-circulation, and reassortment with other strains in terrestrial poultry generated H5N1 (Guan *et al.*, 1999; Choi *et al.*, 2004), H7N9 (Feng *et al.*, 2013; Gao *et al.*, 2013) and H10N8 (Chen *et al.*, 2014; Qi *et al.*, 2014) viruses also causing zoonotic infections. As noted in the Introduction, numerous mutations in the PB2 subunit of the polymerase have been found to be involved in mammalian adaptation of avian viruses. Here, we focused on the role of PB2 mutations E627K, D701N and S714R in host adaptation of a H9N2 virus.

Several mechanisms have been proposed for how these and other polymerase mutations might promote host adaptation (for reviews, see Gabriel *et al.*, 2013; Cauldwell *et al.*, 2014; Gabriel & Fodor, 2014). Specifically, mutation D701N promotes polymerase transport into the nucleus by exposing a nuclear localization signal (NLS) and adapting PB2 to importin-α1 and -α7 in mammalian cells (Tarendeau *et al.*, 2007; Gabriel *et al.*, 2011). By this mechanism, mutation D701N not only mediates nuclear transport of newly synthesized PB2 monomers (Gabriel *et al.*, 2008), but also initiates nuclear entry of incoming viral ribonucleoprotein (vRNP) (Sediri *et al.*, 2015). Mutation S714R has been proposed to cooperate with D701N in exposing the NLS of PB2, thereby facilitating recognition and

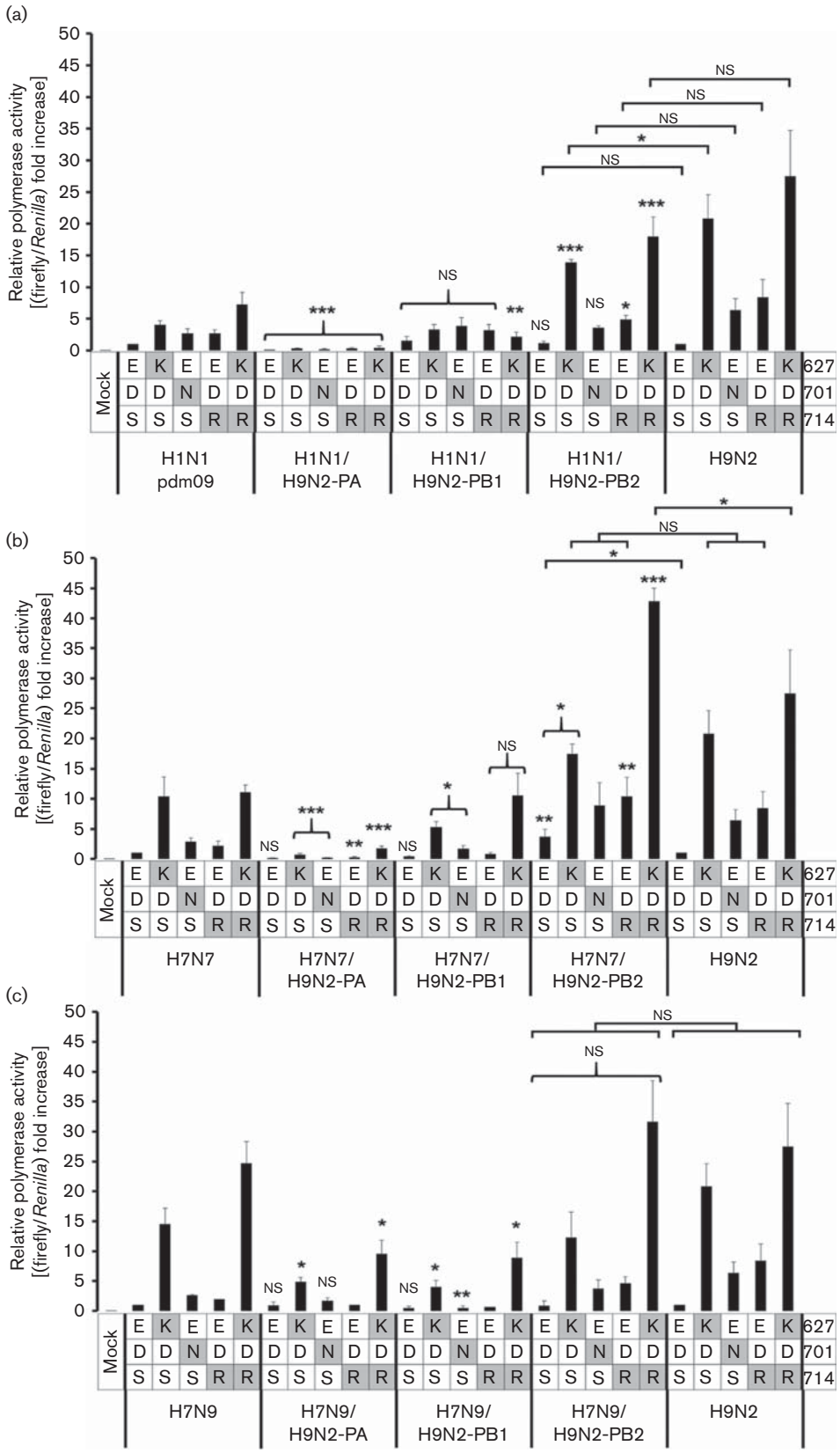

Fig. 5. Impact of PB2 mutations on the activities of polymerase reassortants. HEK 293T cells were transfected with pHW2000 constructs encoding PB1, PA, PB2 and NP, and polymerase activities were determined by dual-luciferase assay.

The activities of (a) H1N1pdm09, (b) H7N7 and (c) H7N9 polymerases and of their reassortants with H9N2-PA, H9N2-PB1 and H9N2-PB2 were analysed for their dependence on the PB2 mutations E627K, D701N and S714R. Activities are indicated relative to the polymerase complex containing the avian signature 627E–701D–714S in PB2 and set to 1. As a background control, plasmids were transfected omitting the PB2 subunit (Mock). The amino acid signatures at positions 627, 701 and 714 of PB2 are shown (grey, mammalian; white, avian). Data represent mean ± SD of at least three independent experiments. Statistical significance of the polymerase increase due to avian/mammalian signature was defined as $P<0.05$ (*$P<0.05$; **$P<0.01$; ***$P<0.001$; NS, non-significant) (Student's *t*-test). The mutants of the H1N1pdm09, H7N7 and H7N9 reassortants were compared with the H1N1pdm09, H7N7 and H7N9 homologous polymerase mutants, respectively. The statistical significance of the activity increase observed with the reassortants containing H9N2-PB2 was assessed by comparison with the H9N2 polymerase mutants (cf. Fig. 1).

nuclear transport by importin-α (Czudai-Matwich *et al.*, 2014). Mutation E627K increases replicative fitness by importin-α binding as well, but by an unknown mechanism not involving nuclear entry of PB2 (Resa-Infante *et al.*, 2008; Hudjetz & Gabriel, 2012). Very recently, evidence has been obtained that mutation E627K has yet another function: it prevents vRNP destabilization of incoming virus by the cytoplasmic pathogen recognition receptor RIG-I and thus supports the onset of infection in mammalian cells (Weber *et al.*, 2015).

In the present study, we show that these mutations promote host adaptation of influenza virus A/quail/Shantou/2061/2000 (H9N2) by enhancing polymerase activity and virus replication in mammalian cells. Furthermore, comparison of the mutations in different influenza strains illustrates that the enhancing effect is more distinct in H9N2 and the related H7N9 polymerase than in the non-related H7N7 and H1N1pdm09 polymerases. Finally, the data demonstrate that the enhancing effect observed in H9N2 polymerase activity is a specific trait of H9N2-PB2.

The effect of the mutations on the biological activities of H9N2 virus are summarized in Table 2. We found that mutation E627K caused a strong enhancement of H9N2 polymerase activity, confirming previous observations (Wu *et al.*, 2009; Li *et al.*, 2012; Wang *et al.*, 2012). Mutations D701N and S714R, to the best of our knowledge analysed here for the first time with a H9N2 virus, increased polymerase activity as well, yet to a lower degree. However, a significant increase was observed with double and triple mutants. The increased polymerase activities were paralleled by enhanced rates of virus replication in Calu-3 cells, supporting the concept that the mutations promote mammalian adaptation of avian influenza A viruses. The effects of the mutations on the pathogenicity of the virus in mice were less pronounced. Mice infected with H9N2 D701N virus did not show signs of illness despite high viral lung titres. Only mice infected with H9N2 E627K virus or the triple mutant showed increased virus titres in the lung, weight loss and disease symptoms.

Comparison of different influenza A viruses indicates that enhancement of the polymerase activity by host adaptive mutations is most distinct with H9N2 and H7N9 viruses. This observation is in line with the close phylogenetic relationship of the polymerases of both viruses (Feng *et al.*, 2013). The mutations analysed here have a relatively low effect when introduced into the polymerase of the H1N1pdm09 virus. This observation is not surprising either, as the H1N1pdm09 polymerase has already been adapted to the human host by mutation Q591R (Mehle & Doudna, 2009), whereas H9N2-PB2 has retained the avian signature at this position (Table 1). Remarkably, however, the effect of the mutations on the polymerase activity of the SC35 (H7N7) virus is low as well when compared with the H9N2 polymerase, although both viruses are of avian origin. These observations suggest that the adaptive effect of the PB2 mutations analysed here is particularly high with the H9N2 and related H7N9 virus, further supporting the concept that these viruses have high pandemic potential.

Table 2. Effects of the adaptive mutations on the biological activities of H9N2 virus

Mutation	Polymerase activity (x-fold increase)	Growth rate (x-fold increase)	Weight (% decrease)	Pathogenicity	Lung titre (p.f.u. ml^{-1})
E627K	20	100	10	+	1×10^5
D701N	6	500	0	0	5×10^4
S714R	8	7	5	0	4×10^4
E627K–D701N	20	100	ND	0	ND
E627K–S714R	27	100	ND	0	ND
E627K–D701N–S714R	38	1000	20	+++	5×10^4

ND, Not determined.

As noted above, several influenza A viruses that emerged in recent years possess genes encoding internal proteins which originally were derived from H9N2 viruses by reassortment (Guan *et al.*, 1999; Choi *et al.*, 2004; Feng *et al.*, 2013; Gao *et al.*, 2013; Chen *et al.*, 2014; Qi *et al.*, 2014). It was therefore of interest to analyse the compatibility of the polymerase subunits of the H9N2 virus with the subunits of other strains. Our results demonstrate that in most combinations subunits H9N2-PA and -PB1 negatively affect polymerase activity. The loss of polymerase activity observed upon combination of H9N2-PA with PB1 and PB2 of H1N1pdm09 and H7N7 viruses might result from inefficient endonuclease activity of PA (Naffakh *et al.*, 2000) or a defect in heterotrimer formation (Li *et al.*, 2008). Our data also indicate that the PA subunit of the H9N2 virus is to some extent more compatible with PB1 and PB2 of H7N9 virus than with PB1 and PB2 of H7N7 and H1N1pdm09 viruses. The H9N2-PB1 did not alter the polymerase activity when combined with H1N1pdm09-PA and PB2, which is consistent with data obtained by Naffakh *et al.* (2000) who proposed that PB1 of avian origin cooperates efficiently with human PB2, PA and NP (nucleoprotein). Quite remarkably, the adaptive mutations enhanced polymerase activity most efficiently when introduced into H9N2-PB2, irrespective of the origin of the other subunits. This indicates that the high susceptibility of H9N2-PB2 to the mutations analysed here does not depend on the other polymerase subunits. In conclusion, the data of this study support the concept that, because of its high adaptive potential, H9N2-PB2 may be an important pandemic risk factor.

METHODS

Viruses. The influenza viruses used in this study were A/quail/Shantou/2061/2000 (H9N2), A/Hamburg/05/2009 (H1N1pdm09), A/Anhui/1/2013 (H7N9) and the chicken-adapted SC35 variant of A/seal/Massachusetts/1/1980 (H7N7) (Li *et al.*, 1990). All viruses were propagated in MDCKII cells with infection medium containing tosyl-phenylalanyl-chloromethyl-ketone (TPCK)-trypsin (1 μg ml^{-1}; Sigma). Cell supernatants were cleared by low-speed centrifugation and stored at −80 °C.

Cells. Human embryonic kidney (HEK 293T), Madin–Darby canine kidney (MDCKII) and human lung adenocarcinoma epithelial (A549) cell lines were cultivated in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10 % FCS (Gibco), 1 % glutamine, 1 % penicillin and 1 % streptomycin. Human airway epithelial cells (Calu-3) were cultivated in DMEM F12 (Gibco) supplemented with 10 % FCS, glutamine, penicillin and streptomycin. All cell growth incubations occurred at 37 °C and 5 % CO_2. For infection, growth medium was changed to infection medium containing 0.3 % BSA (PAA) instead of FCS.

Plasmids. To clone all eight gene segments of H9N2, each viral segment was isolated using a QIAamp viral RNA Mini kit (Qiagen), and reverse transcribed and amplified by using a OneStep RT-PCR kit (Qiagen) with universal primers described elsewhere (Hoffmann *et al.*, 2000). The cDNAs were cloned into the pHW2000 plasmid using *Bsm*BI restriction sites. pHW2000 constructs encoding the polymerase subunit of H7N7 (Gabriel *et al.*, 2005), H1N1pdm09 and H7N9 were cloned following the protocol used for H9N2. Mutations E627K, D701N and S714R were introduced into pHW2000 constructs encoding the PB2 subunit of H9N2 by site-directed mutagenesis using a QuikChange II site-directed mutagenesis kit (Agilent) according to the manufacturer's protocol. Presence of mutations was verified by sequencing.

Minigenome assay. HEK 293T cells were transfected using a ProFection mammalian transfection system (Promega). For this, pHW2000 vector constructs encoding PB1, PA, NP and PB2 containing the different avian and mammalian signatures were transfected together with the reporter constructs pPolI-NP-Luc (kindly provided by Dr T. Wolff, Robert Koch Institute, Berlin, Germany) and pGL4.73 (Promega), encoding the firefly and *Renilla* luciferase, respectively. Firefly and *Renilla* luciferase activities were measured 48 h after transfection using Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

Generation of recombinant viruses. Recombinant H9N2 viruses were generated by reverse genetics according to Hoffmann *et al.* (2000). Briefly, HEK 293T cells were transfected with pHW2000 constructs (1 μg) encoding the eight viral gene segments for 6 h using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Then transfection medium was replaced by fresh DMEM medium containing TPCK-trypsin (1 μg ml^{-1}). After 48 h of incubation, supernatants were collected, treated for 1 h with TPCK-trypsin (10 μg ml^{-1}) at 37 °C and then transferred onto MDCKII cells for another 48–72 h incubation at 37 °C, 5 % CO_2 for virus propagation. All experiments with recombinant H9N2 WT and mutant viruses were approved by the relevant German authorities (Regierungspräsidium Giessen and Behörde für Stadtentwicklung und Umwelt Hamburg) and conducted in Biosafety Level 3 facilities at the Institute of Virology of the University of Marburg or the Heinrich Pette Institute in Hamburg.

Viral growth kinetics. Confluent Calu-3 were infected with recombinant viruses at m.o.i. 10^{-4} and incubated in serum-free DMEM at 37 °C, 5 %CO_2. At 10, 24, 48 and 72 h p.i., supernatants were collected and viral titres (p.f.u. ml^{-1}) determined by plaque assay on MDCKII cells.

Plaque assay. Virus titres were determined by plaque assay with Avicel overlay as described by Matrosovich *et al.* (2006). Briefly, MDCKII cells were inoculated with 10-fold serial dilutions of each sample and incubated with Avicel overlay containing TPCK-trypsin (1 μg ml^{-1}) for 48 h. Following this incubation, cells were fixed with 4 % paraformaldehyde in 1 × PBS, permeabilized with 0.3 % Triton X-100, and immunostained using a rabbit antiserum raised against H9N2 (Institute of Virology, University of Marburg) and an HRP-conjugated secondary antibody (Dako) followed by final incubation with the peroxidase substrate True Blue (KPL).

Animal experiments. Animal experiments were performed according to the guidelines of the German animal protection law at the Heinrich Pette Institute. All animal protocols were approved by the relevant German authority (Behörde für Stadtentwicklung und Umwelt Hamburg). Female BALB/c mice (6–8 weeks old) were anaesthetized intraperitoneally with ketamine/xylazine (100/10 mg kg^{-1}) and infected intranasally with 10^6 p.f.u. of the respective virus diluted in 50 μl sterile PBS. Control groups received PBS. Weight loss and survival were monitored for 14 days p.i. Upon >25 % weight loss, mice were humanely killed according to the guidelines of the German animal protection law. Virus titres [p.f.u. (g organ weight)$^{-1}$] were determined in lung and brain of three mice per group on day 3 p.i. by plaque assay.

Statistical analysis. Statistical significance of animal survival rates was determined with Prism 5.03 (GraphPad) using the log-rank (Mantel–Cox) test and the Kaplan–Meier survival curves. All other mean, SD and P values were calculated using the unpaired, two-tailed Student's t-test. Statistical significance was defined as $P<0.05$.

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