

The Novel Human Influenza A(H7N9) Virus Is Naturally Adapted to Efficient Growth in Human Lung Tissue

Jessica Knepper,^a Kristina L. Schierhorn,^a Anne Becher,^b Matthias Budt,^a Mario Tönnies,^c Torsten T. Bauer,^c Paul Schneider,^d Jens Neudecker,^e Jens C. Rückert,^e Achim D. Gruber,^f Norbert Suttrop,^b Brunhilde Schweiger,^a Stefan Hippenstiel,^b Andreas C. Hocke,^b Thorsten Wolff^a

Division of Influenza and Other Respiratory Viruses, Robert Koch-Institut, Berlin, Germany^a; Department of Internal Medicine/Infectious Diseases and Respiratory Medicine, Charité, Universitätsmedizin Berlin, Berlin, Germany^b; HELIOS Clinic Emil von Behring, Department of Pneumology and Department of Thoracic Surgery, Chest Hospital Heckeshorn, Berlin, Germany^c; Department for General and Thoracic Surgery, DRK Clinics, Berlin, Germany^d; Department of General, Visceral, Vascular and Thoracic Surgery, Universitätsmedizin Berlin, Charité Campus Mitte, Berlin, Germany^e; Department of Veterinary Pathology, College of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany^f

J.K. and K.L.S. contributed equally to this work.

ABSTRACT A novel influenza A virus (IAV) of the H7N9 subtype has been isolated from severely diseased patients with pneumonia and acute respiratory distress syndrome and, apparently, from healthy poultry in March 2013 in Eastern China. We evaluated replication, tropism, and cytokine induction of the A/Anhui/1/2013 (H7N9) virus isolated from a fatal human infection and two low-pathogenic avian H7 subtype viruses in a human lung organ culture system mimicking infection of the lower respiratory tract. The A(H7N9) patient isolate replicated similarly well as a seasonal IAV in explanted human lung tissue, whereas avian H7 subtype viruses propagated poorly. Interestingly, the avian H7 strains provoked a strong antiviral type I interferon (IFN-I) response, whereas the A(H7N9) virus induced only low IFN levels. Nevertheless, all viruses analyzed were detected predominantly in type II pneumocytes, indicating that the A(H7N9) virus does not differ in its cellular tropism from other avian or human influenza viruses. Tissue culture-based studies suggested that the low induction of the IFN- β promoter correlated with an efficient suppression by the viral NS1 protein. These findings demonstrate that the zoonotic A(H7N9) virus is unusually well adapted to efficient propagation in human alveolar tissue, which most likely contributes to the severity of lower respiratory tract disease seen in many patients.

IMPORTANCE Humans are usually not infected by avian influenza A viruses (IAV), but this large group of viruses contributes to the emergence of human pandemic strains. Transmission of virulent avian IAV to humans is therefore an alarming event that requires assessment of the biology as well as pathogenic and pandemic potentials of the viruses in clinically relevant models. Here, we demonstrate that an early virus isolate from the recent A(H7N9) outbreak in Eastern China replicated as efficiently as human-adapted IAV in explanted human lung tissue, whereas avian H7 subtype viruses were unable to propagate. Robust replication of the H7N9 strain correlated with a low induction of antiviral beta interferon (IFN- β), and cell-based studies indicated that this is due to efficient suppression of the IFN response by the viral NS1 protein. Thus, explanted human lung tissue appears to be a useful experimental model to explore the determinants facilitating cross-species transmission of the H7N9 virus to humans.

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Address correspondence to Thorsten Wolff, WolffT@RKI.de.

At least 135 people have been infected with a novel influenza A(H7N9) virus since February 2013 in Eastern China, resulting in a high frequency of severe lower respiratory tract infections and 44 fatalities (1, 2). This novel influenza A virus (IAV) most likely emerged from a zoonotic reservoir, as closely related viruses were isolated from apparently healthy poultry in those provinces (3). Genome sequencing indicated that the H7N9 virus derives its genes from at least three different low-pathogenic avian IAV strains (1, 4, 5). Affected patients experienced febrile influenza-like illness, rapidly progressing to pneumonia and acute respiratory distress syndrome in many cases, indicating the spread of the virus to the lung (1). The

capacity to infect the lower respiratory tract was also reproduced in experimental infections of ferrets, pigs, macaques, and mice, which is unusual for an avian influenza virus (6–9). Recent analyses detected significantly increased cytokine and chemokine levels in A(H7N9) patient serum samples, which may reflect a dysregulation of the immune response contributing to the severity of the disease (10). Although many A(H7N9) patients had underlying chronic conditions, this outbreak challenges the concept that IAV with low pathogenicity in birds infect humans very rarely and do not cause severe disease (11), raising questions as to the specific properties of this novel zoonotic pathogen in humans.

Genetic analyses showed that novel H7N9 viruses harbor adaptive changes associated with enhanced fitness of avian IAV in human hosts. This includes a glutamine-to-leucine change at position 226 (H3 numbering) within the receptor binding site of the viral hemagglutinin, which most likely extends the spectrum of virus receptors by enabling binding to avian (α -2,3-linked sialic acid) as well as human (α -2,6-linked sialic acid) receptor determinants (8, 10, 12, 13). Moreover, the presence of lysine and asparagine at polymorphic amino acid positions 627 and 701, respectively, in the PB2 protein of patient H7N9 isolates indicates initial adaptation for increased polymerase activity in mammalian cells (3). However, virulence of IAV is a multigenic trait, and additional genetic changes encoding adaptive amino acids may be present in the novel H7N9 reassortant virus (4). An important aspect of IAV pathogenicity is the capacity to suppress the innate antiviral response by means of the viral NS1 protein. This multifunctional viral factor limits expression of type I interferon (IFN- α) genes via blockade of the signaling pathway governed by retinoic acid-induced gene I (RIG-I), silences activation of the kinase PKR, and prevents fast apoptosis of infected host cells (14). NS1 proteins of human and avian IAV strains have a length of 215 to 237 amino acids and can differ in their primary sequences by more than 30%, indicating specific adaptations to the respective host environment (15). We previously showed that a low-pathogenic avian IAV (subtype H12N5) was strongly attenuated for growth in explanted human lung tissue but provoked a strong cytokine response in comparison to human-adapted seasonal and pandemic IAV (16). Given the substantial viral loads detected in lower respiratory tract samples of A(H7N9) patients (3), we herein compared the replicative potential and activation of innate responses by an H7N9 patient isolate and two low-pathogenic H7 subtype viruses of the Eurasian lineage in human alveolar tissue.

Replication analysis in primary human lung tissue showed that the A/Anhui/1/2013 (H7N9) virus propagated similarly well as the prototypic human A/Panama/2007/1999 (H3N2) strain over a 48-h period, reaching titers in the range of 10^5 PFU/ml. In contrast, there was hardly any titer increase detectable for two low-pathogenic avian H7 subtype viruses [A/turkey/Italy/472/1999 (H7N1) and A/turkey/Germany/R11/2001 (H7N7)] after inoculation of tissue from identical donor lungs (Fig. 1A). The differences between human and avian H7 viruses became highly significant through the course of infection. Importantly, lung tissue infected with the two avian H7 viruses released significantly higher levels of antiviral IFN- β into the supernatants than the human H7 virus (Fig. 1B). The levels of secreted MIP-1 β , IP-10, and interleukin 1 β (IL-1 β) were also slightly higher for the two avian strains, but those differences were statistically not significant (Fig. 1C and D and data not shown). Differences in viral growth and cytokine induction between avian and human H7 strains might be due to differences in the principal target cells. Therefore, we examined the cellular tropism of the H7-subtype viruses by double staining for viral antigen and cell-type-specific markers in the alveolar tissue. This analysis showed that the human and the avian H7 strains were detected in type II but not in type I pneumocytes (Fig. 1E and data not shown), indicating that those viruses target the same host cell type as was previously shown for other avian, porcine, and human IAV (16).

Growth curve analyses in mammalian and avian cell lines demonstrated that the human H7N9 strain propagated more efficiently than the avian H7 viruses in human (A549) and canine

(MDCK) cells (Fig. 2A and C), whereas there was a more equal growth kinetic in chicken DF1 fibroblasts (Fig. 2B). This result points again toward an unusually high inherent capacity of the H7N9 virus to propagate in mammalian cells. Since the two avian H7 strains provoked significantly higher levels of IFN- β in the lung cultures, we next evaluated activation of the human IFN- β promoter and the suppression of this response by the NS1 proteins expressed by the different strains. Figure 2D illustrates that NS1-deficient human virus strongly activated a stably integrated human IFN- β promoter by more than 45-fold, whereas the isogenic wild-type virus stimulated the promoter only by about 3-fold, illustrating the pivotal role of NS1 in IFN suppression (Fig. 2D). The three H7 strains induced the IFN- β promoter also to a small extent, but the A/Anhui/1/2013 (H7N9) strain was the least active one. Finally, we tested the capacity of transiently expressed NS1 proteins to block RIG-I-dependent upregulation of the IFN- β promoter in human cells infected with NS1-deficient virus. The analysis showed that expression of the hu-H7 NS1 gene suppressed viral activation of the IFN- β promoter significantly stronger than the avian NS1 proteins, whereas NS1 of the epidemic H3 strain had intermediate activity (Fig. 2E). These results suggest that the NS1 protein of the novel H7N9 virus is a potent inhibitor of the antiviral human IFN response and may thereby contribute to its intriguingly strong replication in human alveolar tissue.

The isolation of avian A(H7N9) virus from a substantial number of pneumonia patients was unexpected and is a major cause of concern, even if the virus has not yet acquired the ability for sustained transmission among humans. A total of 16 of the 17 subtypes of the IAV hemagglutinin are found in feral birds (17). Among humans, only viruses of the H1, H2, and H3 subtypes have circulated to larger extents, rendering most of the human population in principle vulnerable to infection with other subtypes. However, transmission of avian IAV to humans occurs rarely, as those viruses usually lack adaptive changes that would enable efficient propagation and spread among humans (18). Here, we describe an experimental model for the A(H7N9) virus in human alveolar lung tissue infected *ex vivo* that complements recent analyses of this virus in animal models (6–9). As the lung organ culture system revealed clear differences between bona fide avian and human-adapted strains, this approach appears useful to evaluate the zoonotic potential of IAV. Our initial assessment demonstrated that the human A(H7N9) virus is as adapted to efficient growth in human lung tissue as a prototypic epidemic virus strain, whereas this tissue was essentially nonpermissive for two avian H7 isolates. The restriction of the avian viruses is most likely not mediated on the level of virus entry, as infected alveolar cells were easily identified in tissue sections for all strains examined and there was no apparent change in the preference to infect type II pneumocytes. This finding is consistent with the presence of receptors for both human and avian IAV cells in human alveolar tissue and recapitulates that receptor specificity is not a decisive determinant of influenza virus tropism in the lower respiratory tract (16). Productive IAV infection of type II pneumocytes and resulting cytopathic effects are expected to compromise alveolar structural integrity by reducing the production of surfactant and to diminish the repair capacity of the injured lung (19, 20).

The human Anhui/1/2013 virus displays an adaptive lysine (K) residue at PB2 position 627, whereas this position is occupied by a glutamic acid (E) in the avian H7 strains. Notably, the human H7

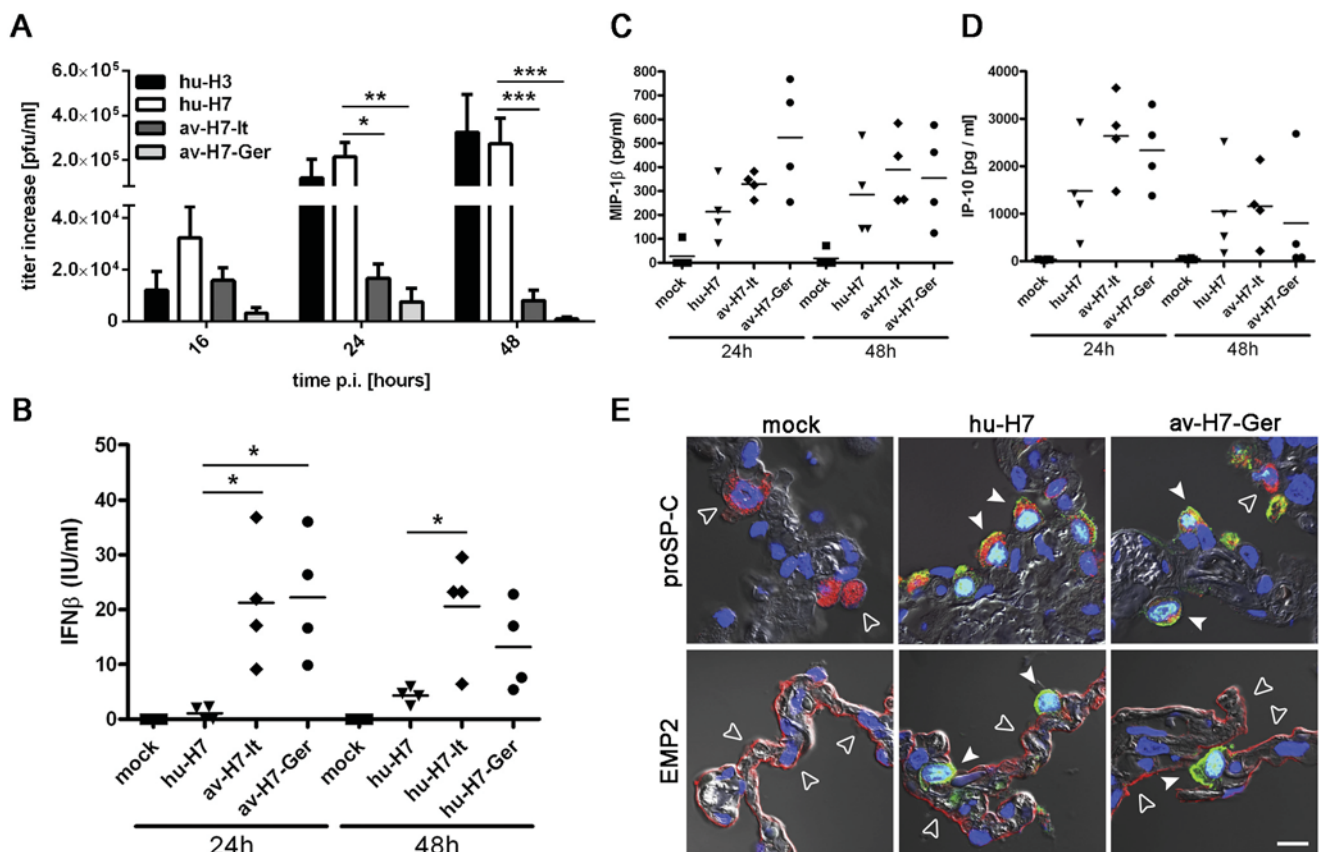


FIG 1 Efficient replication, cytokine induction, and cellular tropism of H7N9 virus in human lung tissue infected *ex vivo*. Tumor-free normal lung tissue was stamped into small cylinders (thickness of ~3 mm, diameter of 8 mm) and incubated in RPMI 1649 medium (containing 0.3% bovine serum albumin [BSA], 2 mM glutamine, and antibiotics) at 37°C with 5% CO₂ as described (16). After overnight incubation, lung organ cultures were inoculated with 4×10^5 (A) or 4×10^6 (B to E) PFU of the influenza A viruses A/Anhui/1/2013 (hu-H7), A/Turkey-Italy/472/1999 (av-H7-It), or A/Turkey-Germany/R11/2001 (av-H7-Ger) for 1 h, followed by being washed with phosphate-buffered saline (PBS) to remove excess virus and incubation in RPMI 1649 medium at 37°C. The epidemic influenza A/Panama/2007/1999 virus (hu-H3) was included as a control. For each experiment, tumor-free tissue specimens from at least three donors were analyzed. Stocks of the avian H7 viruses were grown in embryonated chicken eggs, whereas the human H3 and H7 viruses were propagated in MDCK cells to minimize selection of receptor binding variants. (A) Aliquots of supernatants taken at 0, 16, 24, and 48 h postinfection (hpi) were titrated by standard plaque assay on MDCK cells. The relative growth of the strains is depicted as titer increase compared to the start of infection (0 hpi). The human H7 strain propagated as efficiently as the seasonal human virus, whereas the two avian H7 viruses hardly replicated. Mean values and standard errors of the means (SEM) from 3 independent experiments each done in triplicate are shown. Asterisks indicate significant differences between strains (Mann-Whitney *U* test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (B to D) Aliquots of infected lung culture supernatant taken at 24 or 48 hpi were analyzed for the concentrations of IFN- β (B), MIP-1 β (C), or IP-10 (D) by commercial enzyme-linked immunosorbent assay (ELISA) kits (FUJIREBIO Inc., Invitrogen, BD Biosciences). Data points from four independent experiments are shown individually, and mean values are indicated (line). Significance values between groups are indicated by asterisks (Mann-Whitney *U* test; *, $P < 0.05$). The human H7 virus induced significantly less IFN- β than av-H7-It and av-H7-Ger. (E) Conserved tropism of H7-subtype viruses for type II pneumocytes. Human lung tissue was not infected (mock) or was infected with hu-H7 or av-H7-Ger for 24 h. The tissue samples were fixed and routinely paraffin embedded as described (16). After deparaffinization and antigen retrieval, slices were immunostained with fluorescently (Alexa Fluor 488) labeled anti-influenza A virus antibody (Serotec; OBT1551) (green channel) to detect virus-infected cells (white arrowheads). The slices were costained with rabbit antibodies detecting either pro-SP-C (Chemicon; AB3786; top row) or EMP2 (Sigma; HPA014711; bottom row) to identify type II or type I pneumocytes (red channels), respectively. An Alexa Fluor 594-labeled anti-rabbit F(ab')₂ fragment was applied as the secondary antibody. Nuclei (blue) were counterstained with DAPI (4',6-diamidino-2-phenylindole). Open arrowheads identify noninfected cells. Immunofluorescence was analyzed by spectral confocal microscopy and linear unmixing of tissue autofluorescence by using a 780 laser-scanning microscope (objective, 40 $\times$ /1.30 oil differential interference contrast [DIC] M27). Scale bar, 10 μ m.

strain induced significantly lower levels of antiviral IFN than the avian H7 viruses, which in combination with the PB2 E627K change may explain its robust growth. In fact, our analysis suggests that the NS1 protein encoded by the human H7 isolate is a potent inhibitor of RIG-I-dependent activation of IFN-I genes. The NS1 protein is well known to contribute to influenza A virus pathogenicity and virulence (21, 22). The primary NS1 sequence of the Anhui/1/2013 strain shows conserved residues at positions known to be important for IFN suppression, such as R38 and K41,

but it lacks several other motifs present in NS1 proteins of other IAVs which mediate binding to host factors. This includes the C-terminal 4-amino-acid binding motif for cellular PDZ domain factors (23) as well as residues facilitating interaction with CPSF-30, which is involved in the maturation of cellular transcripts, including IFN- β pre-mRNA (24). Clearly, the significance of specific amino acid residues of the H7N9-Anhui NS1 protein and other viral polypeptides for replication and cytokine control in human respiratory tissues will need to be examined in future re-

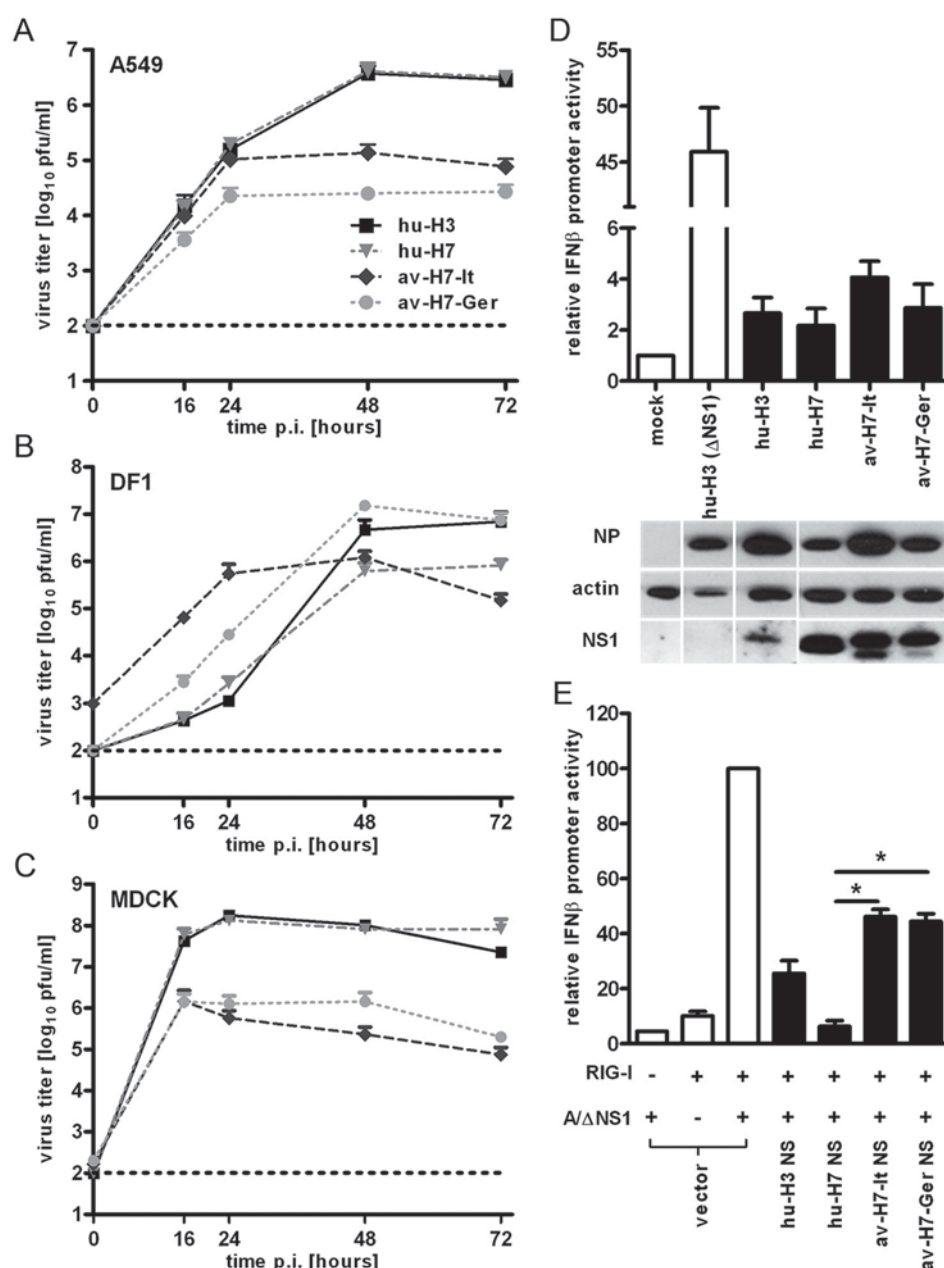


FIG 2 Efficient replication of the H7N9 virus in mammalian cell lines correlates with low activation of the human IFN- β promoter. (A to C) Multicyclic replication of the virus strains A/Anhui/1/2013 (hu-H7), A/Turkey-Italy/472/1999 (av-H7-It), A/Turkey-Germany/R11/2001 (av-H7-Ger), and A/Panama/2007/1999 (hu-H3) was determined in cultures of human A549 lung epithelial cells (A), in chicken DF1 fibroblasts (B), and in canine MDCK epithelial cells (C). Cells were infected with the indicated viruses (multiplicity of infection [MOI] = 0.01) and were incubated at 37°C in the presence of trypsin for 72 h. Aliquots taken at the indicated points were titrated on MDCK cells by using a standard plaque assay. The graphs show mean values and SEM from 3 independent experiments, each done with biological duplicates. Dashed lines indicate the limit of detection. Hu-H7 replicated as efficiently on A549 and MDCK cells as hu-H3, whereas av-H7 viruses were impaired in growth by about 100-fold. On DF1 cells, the viruses showed less variable replication. (D) MDCK cells with a stably integrated human IFN- β promoter luciferase reporter were mock treated or infected with hu-H7, hu-H3, or two av-H7 viruses for 12 h (MOI = 1). We used an isogenic NS1-deleted H3N2 (hu-H3 Δ NS1) mutant virus to determine reporter activation in the absence of a viral IFN antagonist (25). Cells were lysed, and equal amounts of protein were analyzed by luciferase assay (3 experiments conducted in triplicate each, mean $\pm$ SEM; top). Expression of viral NP and NS1 proteins and cellular actin as the loading control was verified by immunoblotting (bottom). Infection with hu-H3 (Δ NS1) caused a strong activation of the IFN- β promoter, whereas a modest induction was observed for all other viruses. (E) NS1 expression plasmids were constructed by placing viral NS cDNA of the indicated strains under the control of the human cytomegalovirus immediate early promoter. Human 293T cells were transfected for 24 h with an IFN- β promoter reporter plasmid, FLAG-RIG-I, and pRL-TK, expressing a renilla luciferase under the control of the constitutive herpes simplex virus (HSV) tk promoter for normalization and an NS1 expression construct from the indicated virus strain as described (25). Empty vector was used as the control. Where indicated, samples were infected for 16 h with NS1-deficient influenza virus (MOI = 1). IFN- β induction was determined by dual-luciferase assays (n = 3 in triplicates, mean $\pm$ SEM; *, P $\leq$ 0.05; Mann-Whitney U test). Hu-H7 NS reduced IFN- β promoter activity significantly stronger than avian H7 strains.

verse genetic analyses. Possibly, only a small number of additional mutations or reassortment events with a human virus strain may increase the potential for enhanced transmission of H7N9-like viruses. Considering the unknown extent of H7N9 circulation in domestic and feral birds and the high case fatality rate in vulnerable humans, it appears important to maintain a high level of surveillance and vigilance for these viruses in the human and animal health sectors.

This study was approved by the ethics committee at the Charité clinic (projects EA2/050/08 and EA2/023/07).

Nucleotide sequence accession numbers. Sequence information for the NS and PB2 segments of the av-H7-It and av-H7-Ger strains has been deposited at the GISAID sequence database (<http://platform.gisaid.org>; accession no. EPI464812, EPI464813, EPI467219, and EPI467220).

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Fresh lung explants were obtained from patients primarily suffering from bronchial carcinoma who underwent lung resection at local thoracic surgery centers. For each experiment, tumor-free tissue samples from at least three donors were used. Written informed consent was obtained from all patients.

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