Genetic Characterization and Zoonotic Potential of Highly Pathogenic Avian Influenza Virus A(H5N6/H5N5), Germany, 2017–2018

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We genetically characterized highly pathogenic avian influenza virus A(H5N6) clade 2.3.4.4b isolates found in Germany in 2017–2018 and assessed pathogenicity of representative H5N5 and H5N6 viruses in ferrets. These viruses had low pathogenicity; however, continued characterization of related isolates is warranted because of their high potential for reassortment.

During winter 2016–17, outbreaks of highly pathogenic avian influenza (HPAI) virus A(H5N8) clade 2.3.4.4b caused substantial losses in wild water birds and domestic poultry across Europe (*1*–*4*). This virus is related to strains from China and Mongolia and has a high potential for reassortment (*4*–*6*). Genetic and temporal analysis of these isolates revealed multiple reassortant events, indicating multiple independent entries into Europe; the outbreaks in Germany were dominated by 5 independent reassortant groups of HPAI virus H5N8 (*5*). Several outbreaks of HPAI virus H5Nx strains in wild birds confirmed the continued presence of H5 clade 2.3.4.4b in Europe well into the summer of 2017. This virus's high tendency to reassort raised concerns that further reassorted strains could dominate in HPAI outbreaks in Europe or become enzootic in wild bird populations in the future. In this study, we set out to characterize related reassortant viruses of subtype H5N6 or H5N5 isolated in Germany during 2017–2018 and delineate their zoonotic potential in ferrets.

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The Study

Starting in November 2017, H5 HPAI viruses, classified as clade 2.3.4.4b according to their hemagglutinin (HA) segments, carrying N6 segments were detected in the Netherlands (*7*), United Kingdom, Switzerland, and Germany (*8*). We used samples mostly from the outbreaks in Germany collected during December 2017–August 2018 (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/25/10/18-1931-App1.pdf). We sequenced (Appendix) and analyzed these viruses and found they carried a neuraminidase (NA) segment of subtype N6 with a high similarity to low pathogenicity avian influenza (LPAI) viruses identified in Asia during 2015–2017 (Appendix Table 2).

According to a full-genome analysis, these H5N6 viruses represent 2 mosaic reassortants of HPAI virus H5N8 found in Europe during the epizootic of 2016–17 (Figure, panel A). Reassortant group I shares all but the NA segment with viruses from the epizootic of 2016–17 (Appendix Figure 1), and because of distinct homologies in the HA, matrix, and nonstructural protein gene segments (Appendix Figure 1), these viruses were further divisible into 3 subgroups, which we designated Gre-02-17-N6, Tai-12-17-N6, and Kor-12-17-N6 (Figure, panel A). The divergence within this reassortant group might have been caused by genetic drift and would be in line with their temporal and geographic patterns of occurrence (Figure, panel B). In contrast, reassortant group II (designated Ger-12-17-N6; Figure) comprises a more homogeneous group of H5N6 viruses from Western and Central Europe. Reassortant group II is genetically distinguishable from reassortant group I by separate clustering of the polymerase acidic (PA) and polymerase basic 2 (PB2) genes (Appendix Figure 1). Group II viruses were detected in Germany during December 2017–August 2018. Their PA segment is similar to that of the HPAI virus A(H5N8) found in the Netherlands in November and December 2016, and their PB2 segment is similar to that of LPAI viruses in Europe and, to a lesser extent, HPAI H5N5 and H5N8 2.3.4.4b isolates from the epizootic of 2016–17 (Appendix Figure 1). This find-

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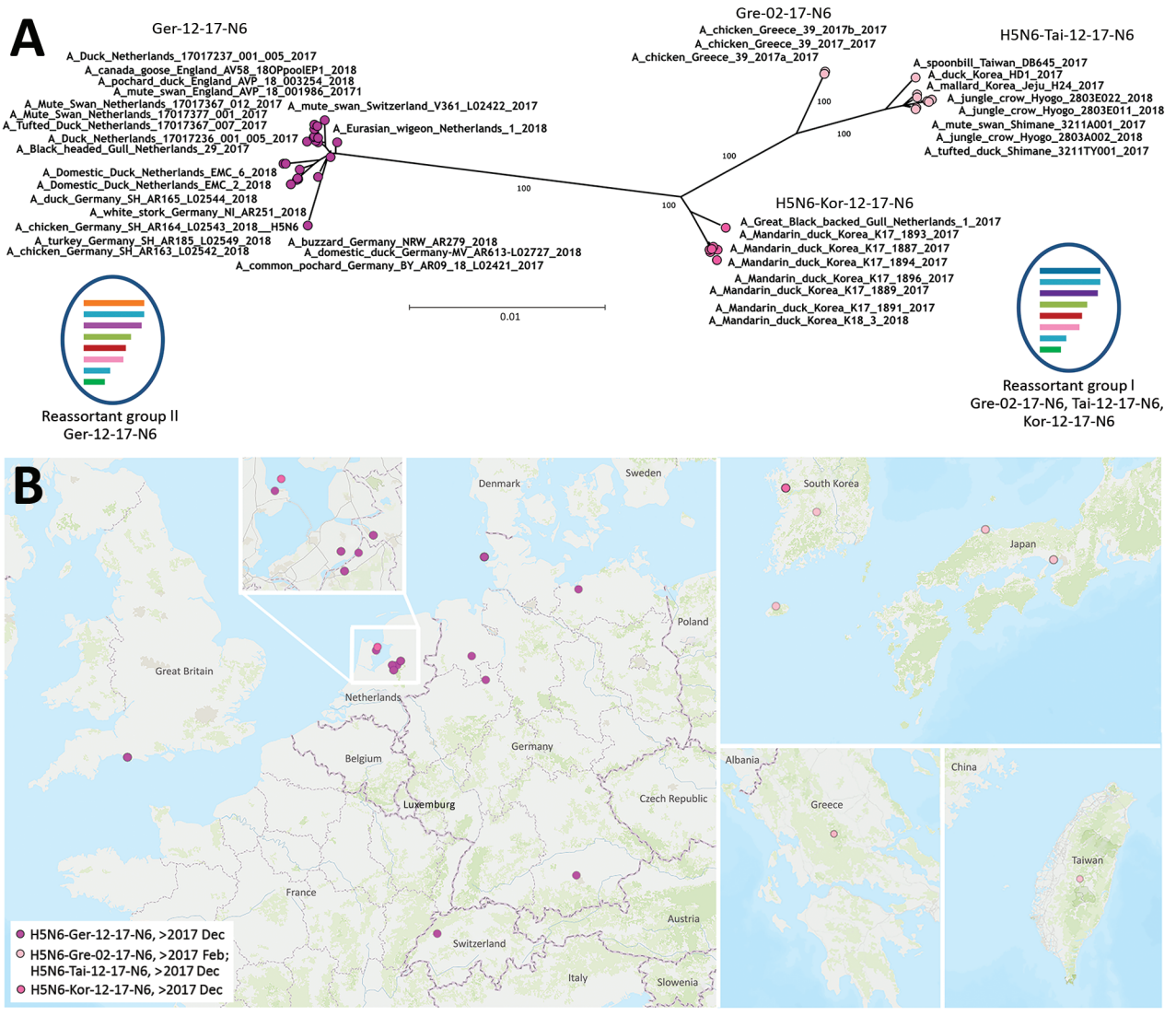

Figure. Phylogenetic clustering and geographic distribution of highly pathogenic avian influenza A(H5N6) viruses, Europe, 2017–2018. A) Supernetwork generated by using maximum-likelihood trees of influenza virus full genomes with RAxML (https://cme.h-its.org/exelixis/web/software/raxml/index.html) and 1,000 bootstrap iterations followed by network analysis with SplitsTree4 (http://ab.inf.uni-tuebingen.de/software/splitstree4). Reassortant viruses are grouped according to their phylogenetic results. Scale bar indicates nucleotide substitutions per site. The mosaic genome structure of reassortant groups I and II is also provided. Gene segment descriptions are given in Appendix Figure 1 (https://wwwnc.cdc.gov/EID/article/25/10/18-1931-App1.pdf). B) Geographic locations of cluster isolates. Inset of cluster in the Netherlands is provided for easier visualization.

ing underscores the ability of HPAI virus clade 2.3.4.4b from the epizootic of 2016–17 to frequently reassort, probably empowered by its genome constellation, especially its HA segment.

H5N6 viruses of clades 2.3.4.4c and 2.3.4.4d and an H5 virus of clade 2.3.4.4b (A/Fujian-Sanyuan/21099/2017) have been reported in cases of human influenza; thus, concerns have been raised about these viruses' zoonotic potential (*9*). Several clade 2.3.4.4b H5 HPAI viruses isolated in South Korea (Appendix Table 3) have already been evaluated in multiple animal models and showed no zoonotic propensity in ferrets (*10*,*11*). These results concur with our previous analysis of cluster 2.3.4.4b HPAI virus H5N8 from Germany (A/tufted_duck_Germany/AR8444/2016) in human lung explants and in ferrets (*12*).

We extended the zoonotic risk assessment of these viruses by using a reassortant group II HPAI H5N6 virus (AR09/18, A/common_pochard/Germany-BY/AR09-18-L02421/2017). For comparison, we included a related reassortant HPAI H5N5 clade 2.3.4.4b virus (AR425/17, A/turkey/Germany-SH/R425/2017) with 3 genes, NA (Appendix Table 2), polymerase basic 1, and nucleoprotein, related to LPAI viruses from different countries and 4 genes, PB2, PA, matrix, and nonstructural protein,

related to HPAI viruses isolated during the epizootic of 2016–17 (*5*).

We inoculated 10 ferrets intranasally with either the H5N6 or H5N5 virus (Appendix). None of the animals displayed any respiratory signs; the only change observed was a minor, short-lived increase in body temperature. Only 1 of the 5 ferrets inoculated with H5N5 exhibited body temperatures >40°C for 3 consecutive days (5–7 days postinfection [dpi]). This particular animal also exhibited a mild gait disorder at 5 dpi, and because these atactic movements persisted (a sign qualifying for termination), the ferret was euthanized at 7 dpi. The viral RNA loads in the nasal washings of animals inoculated with H5N5 and H5N6 were low up through 7 dpi (Table), and RNA excretion ceased thereafter. However, at 7 dpi, the H5N5-inoculated ferret showing mild ataxia displayed a peak of 100 copies/μL of extracted RNA (input volume 100 μL) in the nasal washing fluid (Table).

Nucleoprotein antibody–specific seroconversion (Table) was detected in all inoculated ferrets surviving until euthanasia at 14 dpi. The serum sample of the atactic animal euthanized at 7 dpi scored reactive but not positive.

We dissected all euthanized animals and analyzed spleens, tracheas, lungs, conchae, cerebellum, and cerebrum for viral genome loads, as described previously (*12*). All organ samples taken at 14 dpi were negative; however, the cerebrum, trachea, and nasal concha of the single animal exhibiting disease euthanized at 7 dpi had a low viral load of 7–25 copies/μL of extracted RNA from ≈2 mm^3 tissue material homogenized in 1 mL of medium (input volume 100 μL).

Histopathologic workup of the sick ferret revealed mild, subacute necrotizing rhinitis; moderate, oligofocal, subacute necrotizing bronchointerstitial pneumonia; moderate, multifocal necrotizing hepatitis; severe necrotizing salpingitis; and the focal-to-multifocal intralesional presence of influenza virus matrix protein (Appendix Figure 2) consistent with systemic virus spread. Only 1 of 4 of the H5N5-infected ferrets and 2 of 5 of the H5N6-infected ferrets necropsied at 14 dpi revealed inflammatory lung lesions, yet all were negative for matrix protein by immunohistochemical staining (Appendix Table 4). Considering the low morbidity rate (10%), these H5 viruses have a mild pathogenic potential in the ferret model compared with other HPAI viruses (*13*).

Table. Viral RNA loads in nasal washing samples from ferrets infected with highly pathogenic avian influenza A(H5N6/H5N5) clade 2.3.4.4b virus isolates from Germany, 2017–2018, and seroconversion in study assessing virus zoonotic risk*

	Day postinfection, viral RNA load, genome copies/μL						
Group	0	1	3	5	7	9	Seroconversion†
Controls	–	–	–	–	–	–	–
H5N5	–	0.2	0.2	0.1	100.6	ND‡	+/–§
	–	1.2	2.8	0.1	–	–	+
	–	1.9	0.9	2.9	–	–	+
	–	1.2	–	0.1	–	–	+
	–	0.1	–	8.2	0.9	–	+
H5N6	–	–	0.8	1.3	1.3	–	+
	–	0.2	1.9	13.8	3.2	–	+
	–	0.2	–	0.2	0.2	–	+
	–	–	–	–	–	–	+
	–	0	1.3	0.2	4.2	–	+

*ND, not done; –, negative.
†Seroconversion measured with sensitive nucleoprotein ELISA (ID Screen Influenza A Antibody Competition ELISA Kit; ID.Vet, https://www.id-vet.com) with day 14 or 7 serum sample, depending on day of animal sacrifice, and compared with preinoculation serum sample.
‡Sacrificed day 7 because animal displayed neurologic symptoms.
§Reaction interpreted as reactive but not positive.

Conclusions

The genetic makeup of HPAI H5 clade 2.3.4.4b viruses fosters reassortment, which can expand their evolutionary capacity. Segment reassortment bears a concomitant danger of the emergence of strains that are more pathogenic or zoonotic or that have a higher potential to evolve to propagate in avian hosts with different migratory behaviors. H5N6 and H5N5 viruses of this clade have been continuously present in Europe since 2017, necessitating continuous surveillance and virus characterization. Our study excludes the possibility of enhanced zoonotic potential for the analyzed H5N5 and H5N6 2.3.4.4b clade viruses. Nonetheless, existing reports of clade 2.3.4.4c HPAI H5N6 virus infections in mammals and clade 2.3.4.4b-2.3.4.4d virus co-infections in humans indicate a continued risk for zoonotic events with H5Nx reassortants (*9*). Continued surveillance and characterization of these viruses is crucial to reduce the risk for outbreaks with burgeoning HPAI isolates of the goose/Guangdong lineage.

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Genetic Characterization and Zoonotic Potential of Highly Pathogenic Avian Influenza Virus A(H5N6/H5N5), Germany, 2017–2018

Appendix

Methods

Sequencing and Data Evaluation

RNA of influenza-positive samples was extracted by using Trizol LS (ThermoFisher Scientific, Waltham, USA) and QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). Influenza genome segments were amplified with specific primers by using Invitrogen Superscript III One-Step RT-PCR with Platinium Taq (ThermoFisher Scientific, Waltham, USA). The reverse transcription PCR (RT-PCR) amplicons were sequenced by Sanger or next generation sequencing (NGS) as previously described (*1*,*2*). For NGS, fragmentation of the RT-PCR amplicons was done with a Covaris M220 Ultrasonicator (Covaris Ltd, Brighton, UK) applying a target size of 500 bp. The sonicated cDNA was used for library preparation by using IonTorrent Ion Xpress Barcode Adapters and GeneRead DNA Library L Core Kit (QIAGEN). Size exclusion of the library was done with AMPure XP Magnetic Beads (Beckman Coulter, Fullerton, USA). The libraries were quality checked by using High Sensitivity DNA Chips and reagents on a Bioanalyzer 2100 (Agilent Technologies, Böblingen, Germany), quantized via quantitative PCR with KAPA Library Quantification Kit Ion Torrent (Roche, Mannheim, Germany), and sequenced on an IonTorrent PGM or S5 XL (Thermo Scientific), respectively. Raw sequence data were quality-trimmed and screened for adapter and primer contamination. Consensus sequences were generated with an iterative mapping approach by using Bowtie2 (v. 2.3.0) (*3*) in the Geneious software suite (v. 10.2.3; Biomatters, Auckland, New Zealand). Geneious software suite was also used for quality check and automatic annotation of the sequences. Strain details and epidemiologic information of the viruses sequenced in this study

are given in Appendix Table 1. For network analysis, virus sequences of German high pathogenicity avian influenza (HPAI) H5N6 viruses sequenced in this study (Appendix Table 1) were aligned by segment together with similar sequences from HPAI H5N6 strains aligned by using MAFFT (*4*) (scoring matrix 200PAM/k = 2, gap penalty 1.53, two iterative refinement cycles) receiving 8 alignments, 1 for each segment. Phylogenetic analyzes of these alignments were done by using RAxML (*5*) with general time-reversible plus gamma as the substitution model applying 1,000 bootstrap replicates, resulting in 8 bootstrap-supported phylogenetic trees. All 8 trees were imported into SplitsTree4 for network generation (SuperNetwork, using mean edge weighting, including Z-rule, using Equal Angle for weighted splits transformation, Convex Hull and greedily compatibility applied) (*6*). Maps were created by using ArcGIS Online (www.arcgis.com). Sequences for comparison were retrieved from the Influenza Research Database (www.fludb.org) and EpiFlu Database (www.gisaid.org). We acknowledge the laboratories for providing sequence information via EpiFlu listed in Appendix Table 5. Consensus sequences were published in the EpiFlu Database under accession EPI_ISL_291109, EPI_ISL_291110, EPI_ISL_305453, EPI_ISL_305454, EPI_ISL_305455, EPI_ISL_306989, EPI_ISL_313226, EPI_ISL_313227, and EPI_ISL_322179.

Dataset Selection

The sequence dataset for the phylogeny were selected to include representatives of related strains from the epizootic 2016–2017 and from European and Asian H5N6 outbreaks 2017–2018. In addition, HPAI strains used in animal trials or isolated from humans and, for PB2 phylogeny, similar sequences from low pathogenicity avian influenza viruses are included.

Animal Trial

We tested a HPAIV H5N6 virus (AR09/18; A/common_pochard/Germany-BY/AR09–18-L02421/2017) together with a different reassortant HPAIV H5N5 (AR425/17; A/turkey/Germany-SH/R425/2017) in a ferret inoculation model. The animal experiments gained legal governmental approval through the ethics committee of the State Office of Agriculture, Food Safety, and Fishery in Mecklenburg-Vorpommern (LALLF M-V: LVL MV/TSD/ 7221.3–1.1–023/13). All procedures were carried out in approved biosafety level 3 facilities. We inoculated 5 ferrets (including additional control animals which were not inoculated) per virus subtype intranasally using $10^{5.5}$ 50% tissue culture infectious dose ($TCID_{50}$) per animal of H5N6 AR09/18 (total 75 µL) or 10^{6} $TCID_{50}$ per animal of H5N5 AR425/17 (total 50 µL). Nasal washes

were collected every other day from all ferrets to measure virus excretion by applying 1 mL phosphate-buffered saline into each nostril. Analyses of viral RNA load from nasal washes and organ samples were performed exactly as described (*7*). Ferret serum samples taken preinoculation and at 14 days post infection were heat-inactivated at 56°C for 30 min and analyzed by means of a commercial ELISA for the presence of antibodies against influenza A Virus nucleoprotein (ID Screen Influenza A Antibody Competition ELISA Kit, ID-vet, Montpellier, France) according to the manufacturer´s instructions. Hemagglutination inhibition assays against the homologous H5N5 and H5N6 antigens were performed according to standard protocols (Commission, E. 2006/437/EC: Commission Decision of 4 August 2006 approving a Diagnostic Manual for avian influenza as provided for in Council Directive 2005/94/EC. Report No. ISSN 1725–2555, 16 [2006]).

Strain details and epidemiologic information of the viruses sequenced in this study are summarized in Appendix Table 1. Phylogenetic analyzes of segments were done with similar sequences as described above. The results are given in Appendix Figure 1.

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Name	Subtype	Country	Federal state	Host	Collection date	Reference
A/tufted_duck/Germany/AR8444/2016*	H5N8	Germany	Schleswig-Holstein	*Aythya fuligula*	2016 Nov 7	(*2*)
A/turkey/Germany-SH/R425/2017*	H5N5	Germany	Schleswig-Holstein	Domestic turkey	2017 Jan 22	(*1*)
A/common_pochard/Germany-BY/AR09–18-L02421/2017*	H5N6	Germany	Bavaria	*Aythya ferina*	2017 Dec 28	This study
A/mute_swan/Switzerland/V361-L02422/2017	H5N6	Switzerland	Kanton Bern, Erlach	*Cygnus olor*	2017 Dec 18	This study
A/white_stork/Germany-NI/AR251/2018	H5N6	Germany	Lower Saxony	*Ciconia ciconia*	2018 Apr	This study
A/buzzard/Germany-NRW/AR279/2018	H5N6	Germany	North Rhine-Westphalia	*Buteo*	2018 May	This study
A/chicken/Germany-SH/AR163-L02542/2018	H5N6	Germany	Schleswig-Holstein	Domestic chicken	2018 Mar 19	This study
A/chicken/Germany-SH/AR164-L02543/2018	H5N6	Germany	Schleswig-Holstein	Domestic chicken	2018 Mar 19	This study
A/duck/Germany-SH/AR165-L02544/2018	H5N6	Germany	Schleswig-Holstein	Domestic duck	2018 Mar 19	This study
A/turkey/Germany-SH/AR185-L02549/2018	H5N6	Germany	Schleswig-Holstein	Domestic turkey	2018 Mar 19	This study
A/domestic_duck/Germany-MV/AR613-L02727/2018	H5N6	Germany	Mecklenburg-Vorpommern	Domestic duck	2018 Aug 31	This study

*Used in animal trial.

Appendix Table 2. NA segment similarities (protein and nucleotide) for the N6 and N5 segment of virus A/common_pochard/Germany-BY/AR09–18-L02421/2017 (N6) and A/turkey/Germany-SH/R425/2017 (N5), respectively, compared with similar NA segments from low pathogenicity avian influenza viruses of different subtypes*

Segment	Accession no.	Name	Protein identity	Nucleotide identity
N6	MF694081	A/mallard duck/Georgia/9/2016 (A/H4N6)	457/470 (97%)	1411/1441 (97%)
	MF694113	A/mallard duck/Georgia/3/2016 (A/H4N6)	457/470 (97%)	1412/1441 (97%)
	MH135674	A/duck/Bangladesh/33676/2017 (A/H4N6)	452/470 (96%)	1351/1436 (94%)
	MH071489	A/duck/Bangladesh/24268/2015 (A/H10N6)	450/470 (95%)	1363/1446 (94%)
	LC121366	A/duck/Mongolia/405/2015 (A/H3N6)	450/470 (95%)	1329/1443 (92%)
	LC121262	A/duck/Mongolia/118/2015 (A/H4N6)	450/470 (95%)	1329/1443 (92%)
	KY635728	A/duck/Bangladesh/25891/2015 (A/H4N6)	450/470 (95%)	1356/1446 (93%)
	KY635782	A/duck/Bangladesh/26920/2015 (A/H3N6)	449/470 (95%)	1358/1447 (93%)
N5	KU9629324	A/common redshank/Singapore/F83–1/2015 (A/H9N5)	468/472 (99%)	1398/1418 (98%)
	KP285887	A/migratory duck/Jiangxi/31577/2013 (A/H10N5)	467/472 (98%)	1389/1418 (97%)
	EPI866833	A/duck/Aichi/231002/2016 (A/H6N5)	465/472 (98%)	1383/1423 (97%)
	EPI866951	A/duck/Fukui/181006/2015 (A/H12N5)	462/472 (97%)	1377/1423 (96%)
	KY635758	A/black-tailed godwit/Bangladesh/24734/2015 (A/H7N5)	463/472 (98%)	1386/1423 (97%)
	MF613702	A/shorebird/Delaware Bay/327/2016 (A/H10N5)	454/472 (96%)	1295/1422 (91%)
	MF613865	A/American black duck/Alberta/274/2016 (A/H10N5)	454/472 (96%)	1296/1422 (91%)
	CY240796	A/ruddy turnstone/New Jersey/UGAI16–1448/2016 (A/H10N5)	454/472 (96%)	1299/1427 (91%)

*Typical amino acid markers that are known to support replication in mammalian hosts, for example PB2 627K and PB2 701N (*8*), are not present in the tested viruses and in the first clade 2.3.4.4b human isolate FuSa21099/17. The receptor binding site in the HA segments of the analyzed viruses also indicated a preferred binding to α-2,3 sialic acids present in the avian respiratory tract. Furthermore, H5N8 AR8444/16 and H5N6 AR09/18 NS1 protein do not show a C-terminal PDZ binding motif, which is associated with increased virulence in mice (*8*). HA, hemagglutinin; NA, neuraminidase; PB2, polymerase basic protein 2.

Appendix Table 3. H5Nx-strains tested in the ferret animal model described in this study or in different animal models, and human H5Nx-strains, summarizing their abbreviation, names, and references for their evaluation of zoonotic potential with corresponding relevant genetic markers*

Abbreviation	Name	Subtype	HA recombination binding site	PB2 627	PB2 701	NS PBM	Reference
FuSa21099/17	A/Fujian-Sanyuan/21099/2017	H5N6	QVNGQRG	E	D	GSEV	(*9*)
EMW541/16	A/Environment/Korea/W541/2016	H5N6	QVNGQQG	E	D	ESEV	(*10*)
CTW555/17	A/Common Teal/Korea/W555/2017	H5N8	QVNGQRG	E	D	GSEV	(*10*)
MDK16/16	A/Mandarin duck/Korea/K16–187–3/2016	H5N6	QVNGQQG	E	D	ESEV	(*11*)
AR8444/16	A/tufted_duck_Germany/AR8444/2016	H5N8	QVNGQRG	E	D	†	(*7*)
AR425/17	A/turkey/Germany-SH/R425/2017	H5N5	QVNGQRG	E	D	GSEV	This study
AR09/18	A/common_pochard/Germany-BY/AR09–18-L02421/2017	H5N6	QVNGQRG	E	D	†	This study

*HA, hemagglutinin; NS, nonstructural protein; PB2, polymerase basic 2; PBM, PDZ binding motif.
†NS PBM not present. Truncated protein.

Appendix Table 4. Immunohistological evaluation of tested ferret tissue*

Group	Necropsy, dpi	R.E.	O.E.	Nasolacrimal duct	Sinus maxillaris	Tonsilla palatina	Lungs	Heart	Spleen	Liver	Jejunum	Pancreas	Colon	Kidney	Cerebrum	Cerebellum	M.O.	Other
Neg. control	14	0	ND	ND	ND	0	0	0	0	0	0	0	0	0	0	0	0	
Neg. control	14	0	0	0	ND	0	0	0	0	0	0	0	ND	0	0	0	0	
H5N5	7	0	2	0	0	ND	1	0	0	2	0	0	0	0	0	0	0	1 oral mucosa, 2 ovary, 3 salpinx
H5N5	14	0	0	0	0	0	0	0	0	0	0	0	ND	0	0	0	0	
H5N5	14	0	0	ND	ND	0	0	0	0	0	0	0	ND	0	0	0	0	
H5N5	14	0	0	ND	ND	0	0	0	0	0	0	0	ND	0	0	0	0	
H5N5	14	0	0	0	0	0	0	0	0	0	0	0	ND	0	0	0	0	
H5N6	14	0	0	0	0	0	0	0	0	0	0	0	ND	0	0	0	0	
H5N6	14	0	0	0	0	0	0	0	0	0	0	0	ND	0	0	0	ND	
H5N6	14	0	0	0	ND	0	0	0	0	0	0	ND	ND	0	0	0	ND	
H5N6	14	0	0	0	0	0	0	0	0	0	0	0	ND	0	0	0	0	
H5N6	14	0	ND	0	0	0	0	0	0	0	0	0	ND	0	0	0	0	

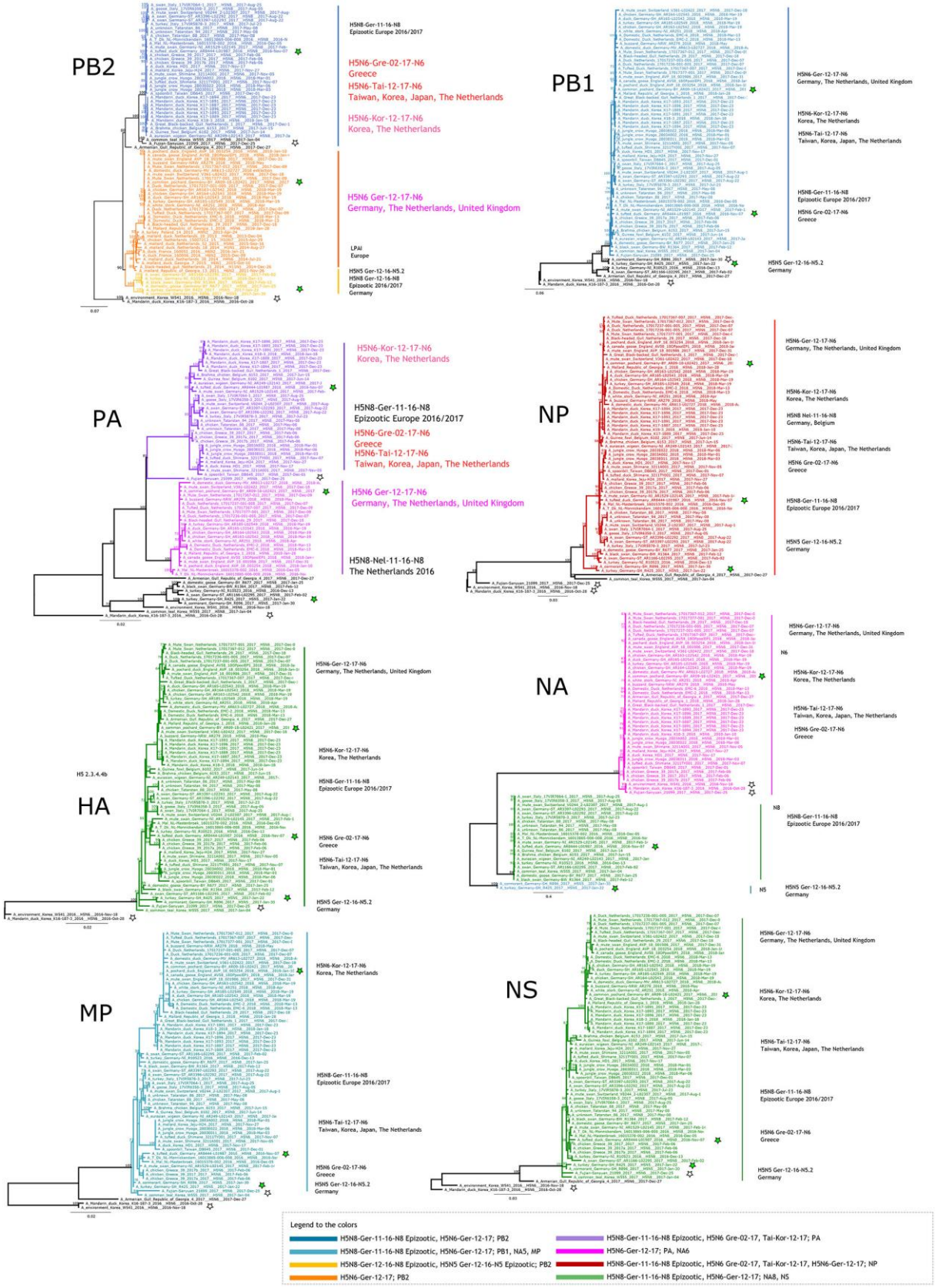
*dpi, days postinfection; ND, not done; neg., negative; M.O., medulla oblongata; O.E., olfactory epithelium; R.E., respiratory epithelium. The distribution of Influenza A virus matrixprotein was semiquantitatively scored as follows: 0 = no immunoreactivity; 1 = focal/oligofocal immunoreactive cells; 2 = multifocal immunoreactive cells; 3 = coalescing/diffuse immunoreactive cells.

Appendix Table 5. Acknowledgment of laboratories providing sequences in EpiFlu database*

Isolate ID	Country	Isolate name	Submitting Laboratory
EPI_ISL_282143	Italy	A/goose/Italy/17VIR6358–3/2017	Istituto Zooprofilattico Sperimentale Delle Venezie
EPI_ISL_289713	The Netherlands	A/Great Black-backed Gull/Netherlands/1/2017	Erasmus Medical Center
EPI_ISL_282141	Italy	A/swan/Italy/17VIR7064–1/2017	Istituto Zooprofilattico Sperimentale Delle Venezie
EPI_ISL_292223	United Kingdom	A/mute_swan/England/AVP_18_001986/2017	APHA
EPI_ISL_288410	The Netherlands	A/Mute Swan/Netherlands/17017377–001/2017	Wageningen Bioveterinary Research
EPI_ISL_288409	The Netherlands	A/Mute Swan/Netherlands/17017367–012/2017	Wageningen Bioveterinary Research
EPI_ISL_273847	Italy	A/turkey/Italy/17VIR5878–3/2017	Istituto Zooprofilattico Sperimentale Delle Venezie
EPI_ISL_287800	Taiwan	A/spoonbill/Taiwan/DB645/2017	Animal Health Research Institute
EPI_ISL_275433	Russia	A/unknown/Tatarstan/94/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_275432	Russia	A/unknown/Tatarstan/86/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_275288	Russia	A/chicken/Tatarstan/88/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_287907	The Netherlands	A/Duck/Netherlands/17017237–001–005/2017	Wageningen Bioveterinary Research
EPI_ISL_287906	The Netherlands	A/Duck/Netherlands/17017236–001–005/2017	Wageningen Bioveterinary Research
EPI_ISL_292225	United Kingdom	A/canada_goose/England/AV58_18OPpoolEP1/2018	APHA
EPI_ISL_288412	The Netherlands	A/Tufted Duck/Netherlands/17017367–007/2017	Wageningen Bioveterinary Research
EPI_ISL_289714	The Netherlands	A/Black-headed Gull/Netherlands/29/2017	Erasmus Medical Center
EPI_ISL_297235	Russia	A/chicken/Rostov-on-Don/1598/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_297234	Russia	A/chicken/Rostov-on-Don/1321/2017	State Research Center of Virology and Biotechnology VECTOR
EPI_ISL_291109	Germany	A/common_pochard/Germany-BY/AR09–18-L02421/2017	Friedrich-Loeffler-Institut
EPI_ISL_292224	United Kingdom	A/pochard_duck/England/AVP_18_003254/2018	APHA
EPI_ISL_312376	Georgia	A/Armenian Gull/Republic of Georgia/4/2017	National Center for Disease Control and Public Health, Georgia
EPI_ISL_289713	The Netherlands	A/Great Black-backed Gull/Netherlands/1/2017	Erasmus Medical Center
EPI_ISL_304404	China	A/Fujian-Sanyuan/21099/2017	Fujian Provincial Center for Disease Control and Prevention
EPI_ISL_288437	South Korea	A/duck/Korea/HD1/2017	Animal and Plant Quarantine Agency (O-2144)
EPI_ISL_288436	South Korea	A/mallard/Korea/Jeju-H24/2017	Animal and Plant Quarantine Agency (O-2144)
EPI_ISL_305417	The Netherlands	A/Domestic_Duck/Netherlands/EMC-6/2018	Erasmus Medical Center
EPI_ISL_305416	The Netherlands	A/Domestic_Duck/Netherlands/EMC-2/2018	Erasmus Medical Center
EPI_ISL_303520	Georgia	A/Mallard/Republic of Georgia/1/2018	National Center for Disease Control and Public Health, Georgia
EPI_ISL_268665	The Netherlands	A/Mal/NL-Mastenbroek/16015378–002/2016	Wageningen Bioveterinary Research
EPI_ISL_268669	The Netherlands	A/T_Dk/NL-Monnickendam/16013865–006–008/2016	Wageningen Bioveterinary Research
EPI_ISL_303643	South Korea	A/Mandarin_duck/Korea/K18–3/2018	Avian Diseases Laboratory, College of Veterinary Medicine, Konkuk University
EPI_ISL_303642	South Korea	A/Mandarin_duck/Korea/K17–1896/2017	Avian Diseases Laboratory, College of Veterinary Medicine, Konkuk University
EPI_ISL_303641	South Korea	A/Mandarin_duck/Korea/K17–1894/2017	Avian Diseases Laboratory, College of Veterinary Medicine, Konkuk University
EPI_ISL_303640	South Korea	A/Mandarin_duck/Korea/K17–1893/2017	Avian Diseases Laboratory, College of Veterinary Medicine, Konkuk University
EPI_ISL_303639	South Korea	A/Mandarin_duck/Korea/K17–1891/2017	Avian Diseases Laboratory, College of Veterinary Medicine, Konkuk University
EPI_ISL_303638	South Korea	A/Mandarin_duck/Korea/K17–1889/2017	Avian Diseases Laboratory, College of Veterinary Medicine, Konkuk University
EPI_ISL_303637	South Korea	A/Mandarin_duck/Korea/K17–1887/2017	Avian Diseases Laboratory, College of Veterinary Medicine, Konkuk University

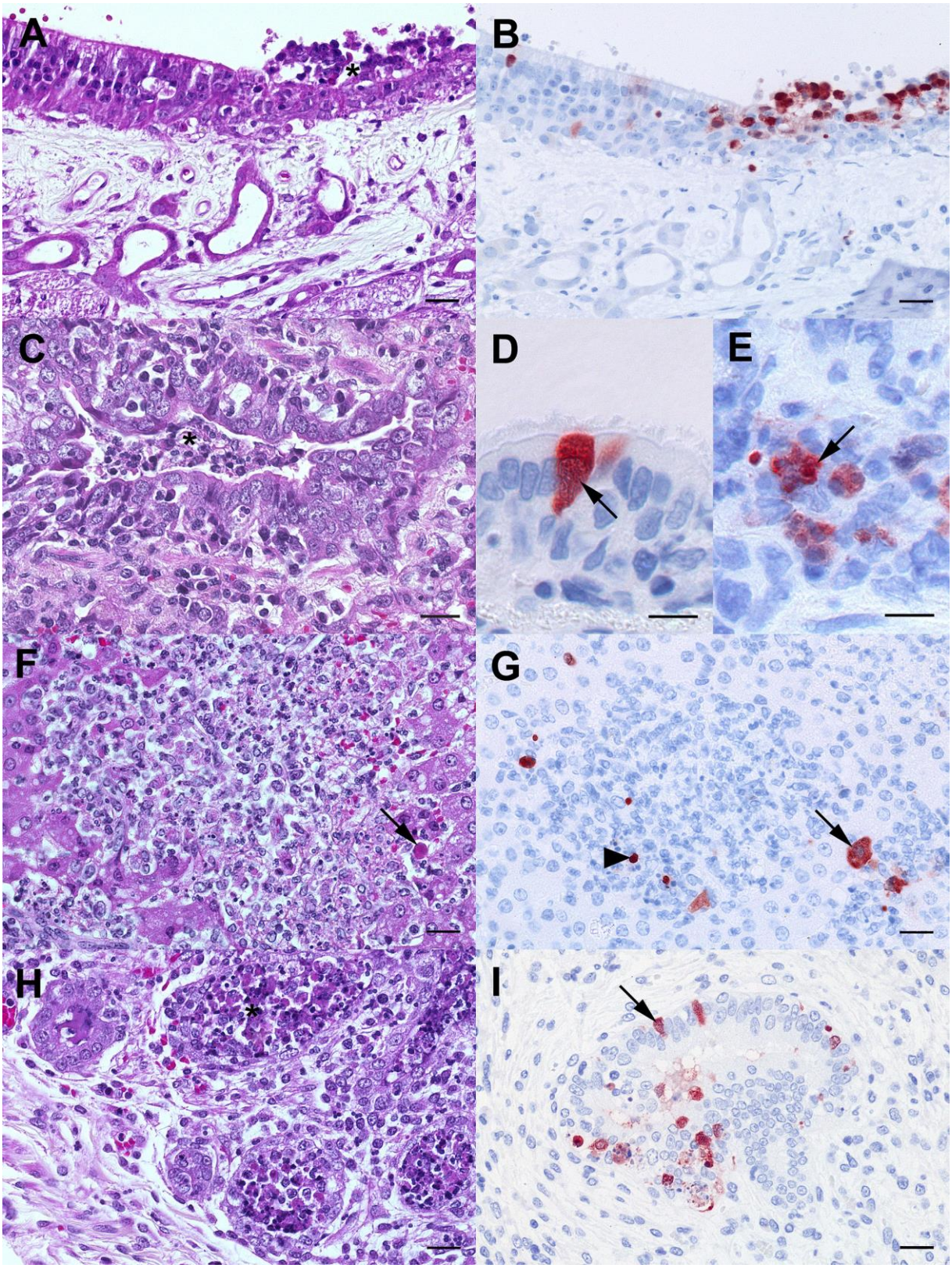
Isolate ID	Country	Isolate name	Submitting Laboratory
EPI_ISL_238148	South Korea	A/Mandarin_duck/Korea/K16–187–3/2016	Avian Diseases Laboratory, College of Veterinary Medicine, Konkuk University
EPI_ISL_288364	Greece	A/chicken/Greece/39_2017b/2017	TVC
EPI_ISL_288363	Greece	A/chicken/Greece/39_2017a/2017	TVC
EPI_ISL_288362	Greece	A/chicken/Greece/39_2017/2017	TVC
EPI_ISL_239393	Japan	A/duck/Aichi/231002/2016	National Institute of Animal Health
EPI_ISL_239416	Japan	A/duck/Fukui/181006/2015	National Institute of Animal Health
EPI_ISL_224753	The Netherlands	A/chicken/Netherlands/15007212/15	Wageningen Bioveterinary Research
EPI_ISL_294779	France	A/duck/France/160051/2016	ANSES
EPI_ISL_294778	France	A/duck/France/160056/2016	ANSES

*ANSES, Agence Nationale de Securite Sanitaire de l'Alimentation, de l'Environnement et du Travail; APHA, Animal and Plant Health Agency; TVC, Thessalonica Veterinary Centre.



Appendix Figure 1. Phylogenetic analyzes of PB2, PB1, PA, HA, NP, NA, MP, and NS genes from H5Nx viruses done by Maximum Likelihood using RAxML. Bootstrap values of 1,000 cycles >50 are included. Scale bars indicate nucleotide substitutions per site. Reassortants are grouped according to phylogenetic results and shown to the right. Stars indicate strains tested in the ferret animal model described in this and a previous study (green stars), strains tested in different animal models (white

stars), and human strains (white stars). H5N6 viruses analyzed in this study are labeled in different colors according to their clusters (see legend). The reassortants H5N8 and H5N5 Germany are described in detail in (*1*). HA, hemagglutinin; MP, matrix protein; NA, neuraminidase; NP, nucleoprotein; NS, nonstructural protein; PA, polymerase acidic protein; PB1, polymerase basic protein 1; PB2, polymerase basic protein 2.



Appendix Figure 2. Histopathological findings in the H5N5-infected ferret necropsied at 7 days postinfection (dpi). A, C, E, G) Hematoxylin eosin. B, D, F, H) Influenza A virus-matrixprotein immunohistochemistry, avidin-biotin-peroxidase complex method, with a monoclonal mouse anti-influenza A virus (strain PR8, A/PR/8/34(H1N1))-matrixprotein immunoglobulin G1 containing hybridoma supernatant (clone M2–1C6–4R3, ATCC HB-64; American Type Culture Collection, Manassas, USA), 3-amino-9-ethyl-carbazol as chromogen and hematoxylin counterstain. A) Nasal

cavity, olfactory mucosa. Mild, focal, subacute, necrotizing rhinitis with degeneration and loss of the olfactory epithelium (asterisk). B) Nasal cavity, olfactory mucosa. Abundant intensely influenza matrixprotein immunoreactive cells and cellular debris within the lesion. C) Lung, bronchioles. Moderate, oligofocal, subacute, necrotizing bronchitis with luminal debris accumulation (asterisk). D) Lung, bronchus. A characteristic bronchial epithelial cell with intense pancellular influenza matrixprotein expression (arrow). E) Lung, bronchiolus. There is intensely influenza matrixprotein immunoreactive cellular debris (arrow) within the lumen of the collapsed and necrotic bronchioles. F) Liver. Moderate, multifocal, subacute, necrotizing hepatitis with infiltrating macrophages, neutrophils, and rare Councilman Corpuscles (arrow). G) Liver. Multifocal intensely influenza matrixprotein immunoreactive intralesional debris (arrow) and fewer hepatocytes (arrowhead). H) Uterine tube. Severe, diffuse, acute, necrotizing salpingitis with nearly complete loss of mucosal epithelium and luminal debris accumulation (asterisk). I) Uterine tube. Multiple influenza matrixprotein immunoreactive epithelial cells (arrow) and debris in luminal recesses in the lamina propria. A–C, F–I) Bar = 20 µm. D, E) Bar = 10 µm.