

Highly Pathogenic Reassortant Avian Influenza A(H5N1) Virus Clade 2.3.2.1a in Poultry, Bhutan

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Highly pathogenic avian influenza A(H5N1), clade 2.3.2.1a, with an H9-like polymerase basic protein 1 gene, isolated in Bhutan in 2012, replicated faster in vitro than its H5N1 parental genotype and was transmitted more efficiently in a chicken model. These properties likely help limit/eradicate outbreaks, combined with strict control measures.

In India and Bangladesh, highly pathogenic avian influenza (HPAI) A(H5N1) viruses of the 2.3.2.1a genetic lineage have circulated in poultry since 2011 (1–3). Subtype H5N1 endemicity is complicated by co-circulating subtype H9N2, G1_Mideast lineage (4,5), which derives 5 internal genes from HPAI A(H7N3) virus from Pakistan (4). A reassortant H5N1 2.3.2.1a virus, rH5N1, with an H9N2-like polymerase basic protein 1 (PB1) gene (H7N3 origin), was reported in Bangladesh (2,5,6), India, and Nepal (7). However, its virulence and transmissibility are undetermined.

In Bhutan, the poultry sector consists of free-range backyard chickens, a rising number of commercial chicken farms, and domestic waterfowl in the south (8,9). Live-bird markets do not exist, but live birds are imported from India (8,9).

Bhutan's poultry sector was severely affected by outbreaks of HPAI A(H5N1) clade 2.3.2.1 virus infection during 2012–2013 (10). Veterinary authorities enforced strict control measures, including depopulation of poultry in affected regions and burning of related housing and equipment (11). Illegal movement of poultry was the major source of outbreaks (11). Although the introduction of

HPAI A(H5N1) from neighboring H5N1-endemic countries is a constant threat, the subtype is not yet entrenched in poultry in Bhutan.

The Study

We isolated HPAI A(H5N1) viruses from samples from 36 chickens and 9 wild birds in Bhutan, all from affected backyard farms adjacent to the highway connecting India with the capital, Thimphu (Figure 1; online Technical Appendix 1 Table 1, <http://wwwnc.cdc.gov/EID/article/22/12/16-0611-Techapp1.pdf>). Phylogenetic analysis (online Technical Appendix 1) suggested that the 2012–2013 outbreaks in Bhutan were caused by the rH5N1 genotype (2.3.2.1a/H9-like PB1 [H7N3 origin]), reported in Bangladesh and India at that time (online Technical Appendix 1 Figures 1, 2; other data not shown). PB1 phylogeny suggested that this genotype underwent 4 independent reassortment events on the Indian subcontinent (online Technical Appendix 1 Figure 2).

Antigenic analysis of selected H5N1 isolates from Bhutan (online Technical Appendix 1) showed homogeneity and a reactivity pattern similar to that of H5N1 reference viruses from Bangladesh (Table). Amino acid differences were observed between strains A/chicken/Bhutan/346/2012 (Ck/Bh/346) (rH5N1) and A/chicken/Bangladesh/22478/2014 (Ck/BD/22478), representing the parental H5N1 clade 2.3.2.1a genotype (pH5N1) (online Technical Appendix 1 Table 2).

To assess whether the rH5N1-PB1 gene conferred a fitness advantage over the pH5N1 genotype, we examined replication kinetics in vitro (online Technical Appendix 1). The replication patterns of rH5N1 and pH5N1 were similar in Madin-Darby canine kidney (mammalian) cells (Figure 2, panel A). However, in chicken embryo fibroblasts (CEFs), Ck/Bh/346 (rH5N1) titers were significantly higher than those of Dk/BD/21326 (rH5N1) ($p < 0.05$) and Ck/BD/22478 (pH5N1) ($p < 0.01$) at 12 hours postinoculation (hpi) and those of Ck/BD/22478 (pH5N1) ($p < 0.001$), and Dk/BD/19097 (pH5N1) ($p < 0.01$) at 24 hpi. Dk/BD/21326 (rH5N1) had significantly higher titers than did Ck/BD/22478 (pH5N1) ($p < 0.01$) at 24 hpi

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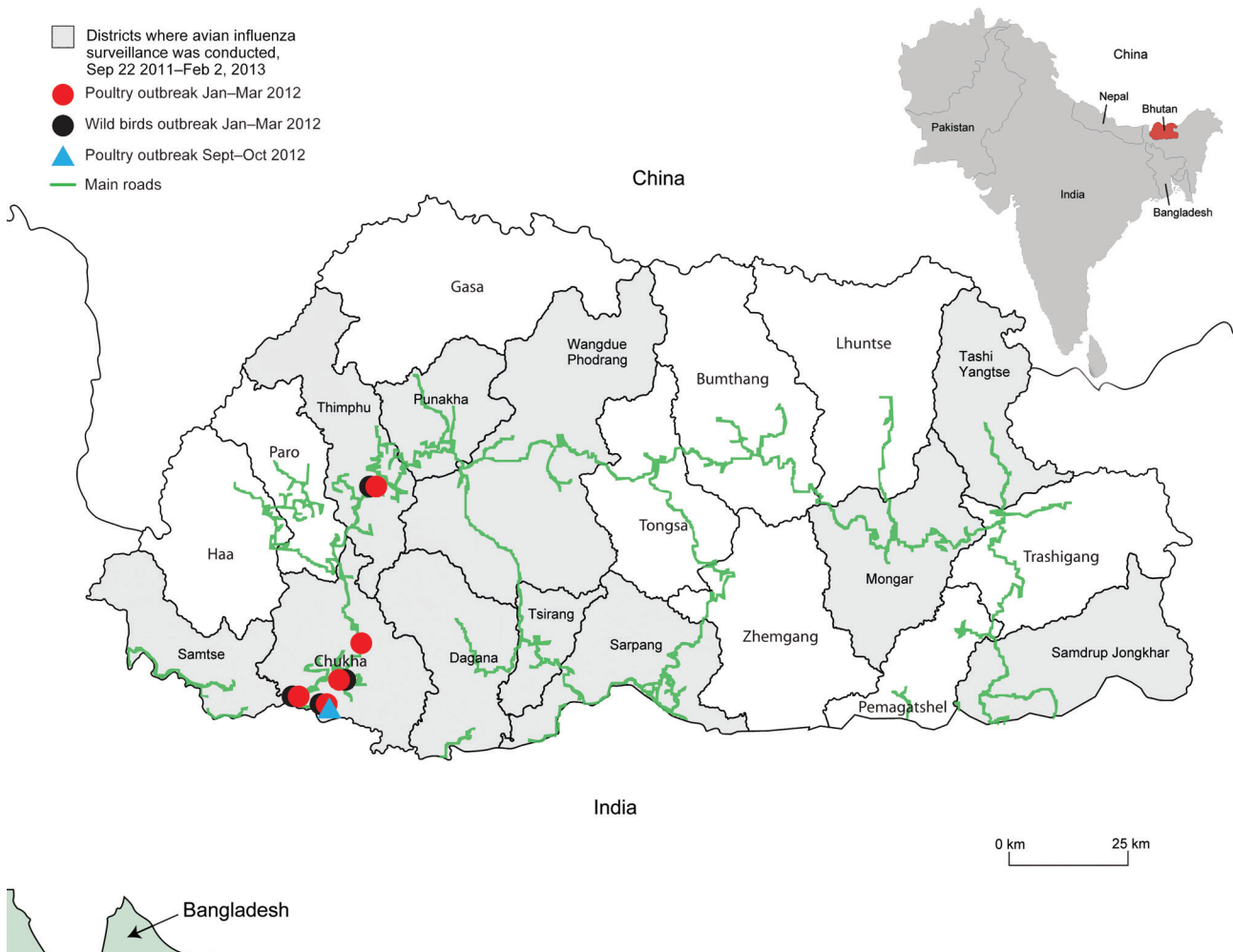


Figure 1. Locations of outbreaks of highly pathogenic avian influenza (H5N1) virus, Bhutan, 2011–2013.

(Figure 2, panel B). These results suggest rH5N1 viruses have a selective growth advantage in avian cells at early time points.

Next, we examined whether this growth advantage reflected higher pathogenicity or transmissibility for Ck/Bh/346 (rH5N1) in chickens than did Ck/BD/22478 (pH5N1) (online Technical Appendix 1). The 50% lethal dose (LD_{50}) for chicken was 16 EID_{50} (50% egg infective dose) for Ck/Bh/346 (rH5N1) and 50 EID_{50} for Ck/BD/22478 (pH5N1). After inoculation with 30 LD_{50} and cohousing with naive contacts, all donors shed virus oropharyngeally and cloacally (Figure 2, panels C, D). All Ck/Bh/346 (rH5N1) donors died within 48 hpi, whereas only 50% of chickens inoculated with Ck/BD/22478 (pH5N1) died. Naive chickens in contact with donors inoculated with Ck/Bh/346 (rH5N1) or Ck/BD/22478 (pH5N1) became infected by day 2 after contact (Figure 2, panel C), started shedding cloacally on day 3 (Figure 2, panel D), and died by day 4. On day 3 after contact,

Ck/Bh/346 (rH5N1) contacts had oropharyngeal and cloacal titers $>1 \log_{10} EID_{50}/mL$ higher than those of Ck/BD/22478 (pH5N1) contacts (Figure 2, panels C, D), but the difference was not significant.

We placed Ck/Bh/346 (rH5N1) and Ck/BD/22478 (pH5N1) in direct competition by co-housing chickens inoculated with each virus with naive contacts (online Technical Appendix 1). All donors shed virus oropharyngeally and cloacally, starting at 1 day postinoculation (dpi). By day 3 after contact, real-time reverse transcription PCR to detect PB1 (online Technical Appendix 1) revealed that 7 of 8 naive contacts simultaneously exposed to both viruses were infected with Ck/Bh/346 (rH5N1) alone, none was infected with Ck/BD/22478 (pH5N1) alone, and 1 was co-infected with both viruses. Thus, despite the lower infectious dose used for 30 LD_{50} , Ck/Bh/346 (rH5N1) killed inoculated chickens faster than did Ck/BD/22478 (pH5N1) and was transmitted faster and more efficiently to naive contacts.

Table. Results of hemagglutination inhibition assays of highly pathogenic avian influenza H5N1 viruses isolated in Bhutan, 2012*

Antigens	Genetic clade	Postinfection ferret antisera				
		α - A/BHG/QH/IA clade 2.2	α - A/Hubei/1/2010 clade 2.3.2.1a	α - A/ck/BD/15205 clade 2.3.2.1a	α - A/dk/BD/19097 clade 2.3.2.1a†	α - A/ck/Bhutan/346 clade 2.3.2.1a†
Reference antigens						
A/BHG/QH/IA	2.2	320	40	40	80	40
A/Hubei/1/2010	2.3.2.1a	40	640	160	1280	80
A/ck/BD/15205	2.3.2.1a	10	80	80	320	40
A/dk/BD/19097	2.3.2.1a	-	40	80	320	40
A/ck/Bhutan/346	2.3.2.1a	10	40	80	640	80
Test antigens						
A/chicken/Bhutan/257/2012	2.3.2.1a	20	40	40	640	40
A/chicken/Bhutan/260/2012	2.3.2.1a	20	40	80	640	80
A/wild bird/Bhutan/357/2012	2.3.2.1a	20	40	80	640	40
A/chicken/Bhutan/1026/2012	2.3.2.1a	40	40	80	1280	80
A/chicken/Bhutan/1030/2012	2.3.2.1a	80	160	320	1280	320
A/chicken/Bhutan/317/2012	2.3.2.1a	10	40	80	640	80
A/wild bird/Bhutan/326/2012	2.3.2.1a	10	80	40	320	20
A/wild bird/Bhutan/328/2012	2.3.2.1a	40	20	40	640	80
A/wild bird/Bhutan/356/2012	2.3.2.1a	40	160	160	640	80
A/chicken/Bhutan/406/2012	2.3.2.1a	20	40	80	320	80
A/chicken/Bhutan/413/2012	2.3.2.1a	20	40	80	640	40
A/chicken/Bhutan/505/2012	2.3.2.1a	80	40	80	640	80
A/chicken/Bhutan/933/2012	2.3.2.1a	40	40	80	640	80
GMT (95% CI)		27.54 (18.36–41.30)	49.51 (34.61–70.83)	80 (56.83–112.6)	640 (502.5–815.1)	68.17 (46.24–100.5)

*Boldface indicates homologous titers. A/BHG/QH/IA, A/bar-headed goose/Qinghai/IA/2005; A/ck/BD/15205, A/chicken/Bangladesh/15205/2012; A/ck/Bhutan/346, A/chicken/Bhutan/346/2012; A/dk/BD/19097, A/duck/Bangladesh/19097/2013; GMT, geometric mean titer.

†The immune response in ferrets was boosted with Freund's incomplete adjuvant (InvivoGen, San Diego, CA, USA) at day 14 postinfection.

We assessed the risk for human infection with rH5N1 by investigating its pathogenicity and transmissibility in ferrets (online Technical Appendix 1). Donors shed $4.5 \log_{10}$ EID₅₀/mL and $3.4 \log_{10}$ EID₅₀/mL in nasal wash samples at 2 dpi and 4 dpi, respectively, but cleared the virus by 6 dpi. No direct or aerosol contacts shed virus, suggesting that Ck/Bh/346 (rH5N1) was not transmitted (data not shown). No Ck/Bh/346 (rH5N1)-inoculated ferrets lost >5% of their body weight or showed elevated body temperature (data not shown). Histopathologic analysis showed that 1 donor, who was lethargic at 3–10 dpi, had mild meningoencephalitis at 14 dpi (online Technical Appendix 2, <http://wwwnc.cdc.gov/EID/article/22/12/16-0611-Techapp2.pdf>). A nucleocapsid protein-positive cell was detected in the brain, suggesting that Ck/Bh/346 (rH5N1) is neurotropic. The other ferrets showed no clinical signs of disease. Virus replication was detected in the lung at 4 dpi (\log_{10} 2.75 EID₅₀/g) (online Technical Appendix 2).

Conclusions

Our study revealed that the viruses that caused the 2012 outbreaks in Bhutan belonged to the rH5N1 genotype (2.3.2.1a/H9-like PB1 [7:1]), whereas neither H9N2 nor the pH5N1 genotype have been detected there. rH5N1 has been isolated sporadically at live-bird markets and from chickens on farms where outbreaks occurred in Bangladesh (5,6), India (12), and Nepal (7) in 2011–2013. The

lack of data on the effect of the H9-like PB1 gene on the virulence of rH5N1 makes determining its pathogenicity and transmissibility a critical public-health goal for Bhutan and neighboring countries.

Ck/Bh/346 (rH5N1) killed inoculated chickens faster than did Ck/BD/22478 (pH5N1), despite the lower infectious dose used for Ck/Bh/346. In CEFs, Ck/Bh/346 replicated with greater efficiency during the first 36 hpi than did Ck/BD/22478, which possibly explains why rH5N1 transmits more efficiently to naive chickens when directly competing with pH5N1. How faster replication contributes to the increased mortality rate of naive chickens might be crucial to eradicating the disease in Bhutan. In a mountainous region with widely separated villages, small-scale poultry farming, and no live-bird markets, the severity and rapid onset of the infection could lead to host-resource exhaustion and self-limitation.

To determine whether the reassortant PB1 gene accounts for the observed phenotypic properties of rH5N1, reverse genetics experiments are required. Despite its enhanced transmissibility, rH5N1 did not supplant pH5N1 in India or Bangladesh after undergoing multiple reassortment events. Possible reasons for this include the involvement of other influenza subtypes, which would complicate the competition/transmission model, especially at live-bird markets, as well as the large duck population, which is prone to inapparent HPAI infection (indicating possible underreporting).

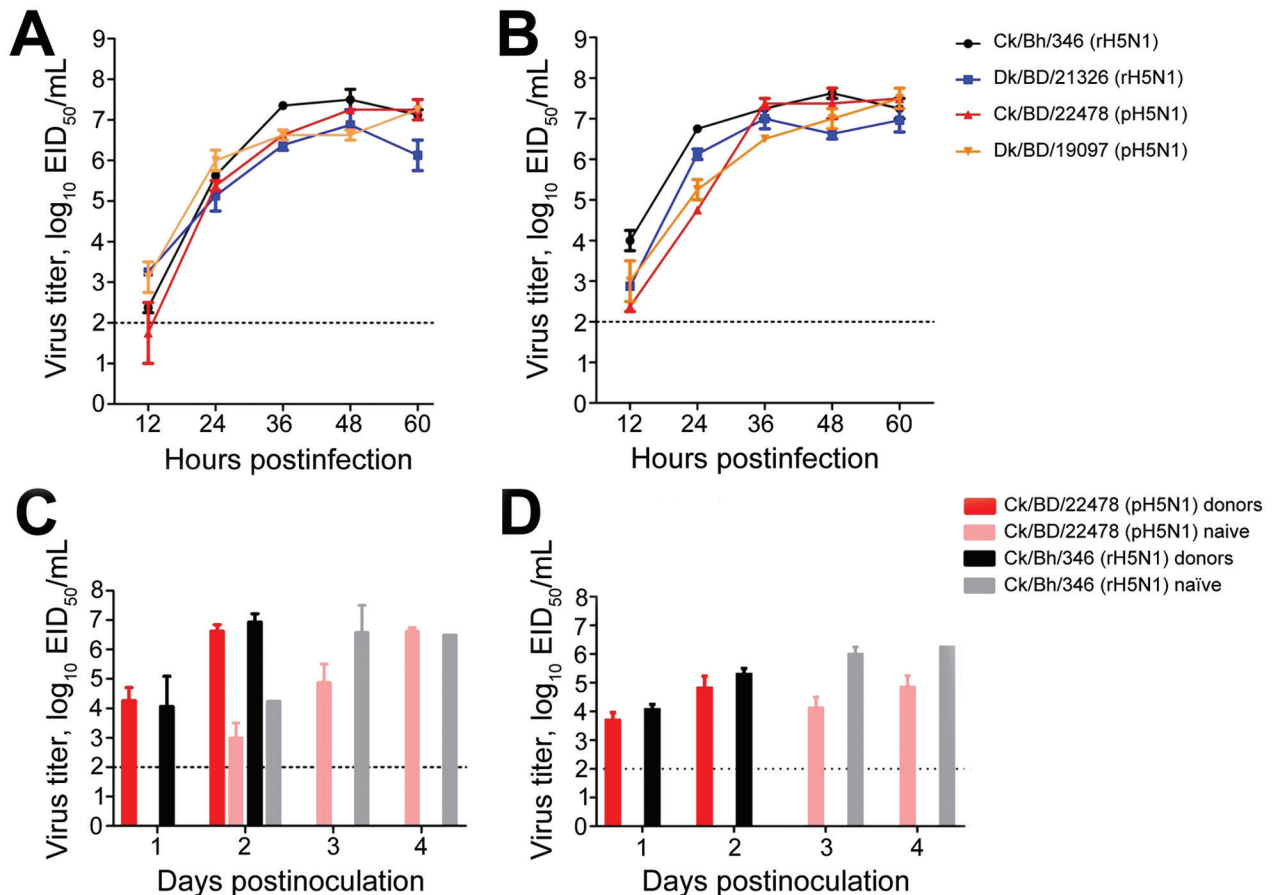


Figure 2. Pathogenesis of influenza virus rH5N1 and pH5N1 2.3.2.1a genotypes in vitro and in vivo. A) Replication kinetics of rH5N1 and pH5N1 in Madin-Darby canine kidney (mammalian) cells. B) Replication kinetics of rH5N1 and pH5N1 in chicken embryonic fibroblast (avian) cells. C) Oropharyngeal shedding and transmissibility of rH5N1 and pH5N1 in a single-virus transmission model in 5-week-old White Leghorn chickens. D) Cloacal shedding and transmissibility of rH5N1 and pH5N1 in a single-virus transmission model in 5-week-old White Leghorn chickens. Naive chickens were co-housed with donors infected with either Ck/22478 (pH5N1) or Ck/Bh/346 (rH5N1) (C and D). The dashed line in each panel represents the limit of virus detection. ANOVA, analysis of variance; Ck/22478, A/chicken/Bangladesh/22478; Ck/Bh/346, A/chicken/Bhutan/346/2012; Dk/BD/23126, A/duck/Bangladesh/23126; Dk/BD/19097/2013, A/duck/Bangladesh/19097; EID₅₀, egg infectious dose; dpi, days postinoculation; hpi, hours postinfection; pH5N1, pandemic H1N1; rH5N1, reassortant H5N1.

Our ferret model results suggest that avian-to-human transmission of rH5N1 is possible. However, further adaptation to the host is necessary for rH5N1 to become transmissible among mammals. Similar results have been reported for H5N1 clade 2.3.2.1 (13), H5N1 clade 2.3.4 (14), and H5Nx clade 2.3.4.4 (15). rH5N1 is potentially neurotropic, manifesting clinically as mild meningoencephalitis with no obvious respiratory involvement. This finding has implications on early diagnosis and use of antiviral drugs during the first 48 hours after clinical diagnosis for optimal therapeutic effect.

pH5N1 and H9N2 virus strains will likely continue to co-circulate on the Indian subcontinent, enabling further reassortment events. Our results highlight the need for active surveillance and full-genome sequencing of all H5N1 virus isolates.

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Highly Pathogenic Reassortant Avian Influenza A(H5N1) Virus Clade 2.3.2.1a in Poultry, Bhutan

Technical Appendix 1

All experiments were conducted in enhanced animal biosafety level 3 (ABSL3+) facilities at St. Jude Children's Research Hospital (St. Jude, Memphis, TN, USA) and were approved by the institutional Animal Care and Use Committee.

Sample Collection and Virus Isolation

From September 22, 2011, to February 2, 2013, oropharyngeal and cloacal swab samples from poultry (1,005 chickens and 41 ducks) and 698 fecal samples from wild birds (including the black-necked crane [*Grus nigricollis*], the cattle egret [*Bubulcus ibis*], the yellow-billed duck [*Anas undulata*], and other terrestrial wild birds) were collected from 11 districts in Bhutan (Figure 1). Samples were stored in 1 mL of glycerol medium (1), transported to St. Jude, and injected into 10-day-old embryonated chicken eggs for virus isolation (2). The presence of influenza A virus (IAV) in allantoic fluids testing positive for hemagglutination was confirmed by endpoint real-time reverse transcription PCR (RT-PCR) (to detect the M gene) and sequencing (3).

Serologic Testing

Fourteen representative subtype H5N1 viruses from Bhutan were selected for use in hemagglutination-inhibition (HI) tests (4), on the basis of their genetic relationships (nucleotide differences in the hemagglutinin (HA) gene), as well as the location and date of their isolation. Postinfection ferret antisera from A/H5N1 clades 2.2 and 2.3.2.1, including recent isolates from Bhutan and Bangladesh (Table), were produced as described (5). Horse red blood cells (1% + 0.5% bovine serum albumin; Sigma, Saint Louis, MO, USA) were used for HI tests (6).

Viral Genome Sequencing and Phylogenetic Analysis

Full-genome sequencing was performed at the Hartwell Center for Bioinformatics and Biotechnology at St. Jude, as described (5). Gene sequences obtained were submitted to GenBank under accession nos. KJ682226–KJ682315 and KX215199–KX215468. The Lasergene package (DNASTAR, Madison, WI, USA) was used for sequence analysis. Phylogenetic analyses of full-length nucleotide sequences were conducted by the maximum-likelihood method, using the Tamura-Nei nucleotide substitution model with 1,000 bootstrap replicates in the MEGA 5 software (7). Reference sequences of all available rH5N1 viruses from the Indian subcontinent (as of February 1, 2016) were retrieved from GenBank (8) and GISAID EpiFlu (9).

Virus Selection

A/chicken/Bhutan/346/2012 (Ck/Bh/346) was selected as the representative virus, because all rH5N1 isolates were genetically and antigenically homogeneous. For in vivo experiments in chickens, Ck/Bh/346(rH5N1) was paired with A/chicken/Bangladesh/22478/2014 (Ck/BD/22478), which represents the pH5N1 genotype that is circulating widely in the Indian subcontinent. For in vitro replication experiments, 2 viruses of duck origin, rH5N1 A/duck/Bangladesh/21326/2013 (Dk/BD/21326) and pH5N1 A/duck/Bangladesh/19097/2013 (Dk/BD/19097), were added.

Growth Kinetics

Virus replication kinetics was determined in MDCK cells and UMNSAH/DF-1 chicken embryo fibroblasts (CEFs) (ATCC, Manassas, VA, USA). Briefly, MDCK cells and CEFs grown in duplicate wells of 6-well plates were inoculated with Ck/Bh/346(rH5N1), Ck/BD/22478(pH5N1), Dk/BD/21326(rH5N1), or Dk/BD/19097(pH5N1) (at a multiplicity of infection of 0.001 PFU/cell). The supernatant was harvested 12–60 hours postinoculation (hpi) and titrated in embryonated chicken eggs with 50% egg infective doses (EID₅₀). Virus titers were calculated by the Reed and Muench method (10).

Pathogenicity and Transmissibility in Chickens

Five-week-old specific-pathogen-free White Leghorn chickens (3 chickens/group; 6 groups/virus) were used to determine the 50% lethal dose (LD₅₀) of Ck/Bh/346(rH5N1) and Ck/BD/22478(pH5N1) by the Reed and Muench method (10). LD₅₀ titers were expressed as the EID₅₀ value corresponding to 1 LD₅₀.

Chickens ($n = 4$) were inoculated with 30 LD₅₀/0.5 mL of Ck/Bh/346(rH5N1) or Ck/BD/22478(pH5N1) by the oral, intraocular, or intranasal route, and at 1 hpi, donor birds were cohoused with naïve contacts ($n = 2$). Surviving donors were removed from the cage at 48 hpi to avoid further exposure to contact birds. Oropharyngeal and cloacal swabs were collected daily for 4 days and titrated in eggs as described above. Morbidity and mortality were monitored twice daily. Competitive virus transmission was examined in a similar setup, except that 2 donor birds were inoculated with Ck/Bh/346(rH5N1) and the other 2 with Ck/BD/22478(pH5N1). The experiment was conducted in 4 replicates in separate isolators. All chickens were screened daily for the presence of virus.

Genotype Determination by Real-Time RT-PCR

Genotypic analysis of competitive virus transmission was performed by multiplex real-time RT-PCR by using viral RNA extracted from individual swabs. Briefly, a set of primers was designed to match the PB1 gene sequence of both the pH5N1 and rH5N1 genotypes (PB1–1462F 5'-GAAGTCTTACATAAATCGGACAGG-3' and PB1 1640R 5'-GTCCTTGATGAATAGCTGAAGAGC-3'). One probe was designed to match the PB1 sequence of pH5N1 (PB1–1544R 5'-/5HEX/CCACTCCAA/ZEN/AACTGGGCAGCTCCATACTG/3IABkFQ/-3') and another to match the rH5N1 PB1 sequence (PB1 1568R 5'-/56-FAM/CTCATGTCA/ZEN/GCTGACTCGTTAATCCCAGAT/3IABkFQ/-3'). Cycling conditions were as previously described (11). The cutoff cycle threshold (CT) value for rRT-PCR was 40. The specificity of the 2 probes for the viruses used in the study [Ck/Bh/346 (rH5N1), Ck/BD/22478 (pH5N1), Dk/BD/21326 (rH5N1), and Dk/BD/19097 (pH5N1)] was tested. Each probe was able to detect only the RNA of the 2 virus strains that belong to the respective genotype for which it was designed.

Pathogenicity and Transmissibility in Ferrets

Nine 3- to 5-month-old male ferrets (Marshall Farms) that were seronegative by the HI test for currently circulating influenza A/H1N1, H3N2, H5N1, and influenza B viruses were used in the transmission model as previously described (12). Briefly, 3 donor ferrets were inoculated intranasally with 10⁶ EID₅₀/0.5 mL of Ck/Bh/346(rH5N1), then each of them was cohoused with a separate naïve direct-contact and a naïve aerosol-contact ferret. Control ferrets were mock-

inoculated with sterile phosphate-buffered saline (PBS). Nasal wash specimens from all ferrets were collected every other day after inoculation, and the virus was titrated in eggs. Clinical signs of infection, body temperature, and weight were recorded daily for 14 days.

One ferret inoculated with Ck/Bh/346 was euthanized at 4, 6, and 14 days postinoculation (dpi). Nasal turbinates, trachea, lung, heart, thymus, spleen, small and large intestines, brain, cerebellum, and spinal cord were collected and homogenized in PBS with antibiotics. The virus titer (EID_{50}/g tissue) was then determined in eggs. Histopathologic and immunohistochemical analyses to detect the IAV nucleoprotein were performed as described (12,13).

Statistical Analysis

Statistical significance ($p \leq 0.05$) between groups was determined by 2-way analysis of variance (ANOVA), performed by using the Prism 5 software (GraphPad, La Jolla, CA, USA).

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Technical Appendix 1 Table 1. Field data for highly pathogenic reassortant avian influenza A/H5N1 (rH5N1)–positive samples collected in Bhutan in 2012

Isolate no.	Collection date	Location state	Location	Habitat	Isolate name
1	01/08/2012	Thimphu	Thimphu	Farm, free-range	A/chicken/Bhutan/367/2012
2	01/15/2012	Chukha	Bjabchu	Farm, free-range	A/chicken/Bhutan/317/2012
3	01/16/2012	Thimphu	Changgidaphu	Farm, free-range	A/chicken/Bhutan/406/2012
4	01/16/2012	Thimphu	Changgidaphu	Farm, free-range	A/chicken/Bhutan/407/2012
5	01/23/2012	Chukha	N/A	Farm, free-range	A/wild bird/Bhutan/505/2012
6	01/23/2012	Chukha	N/A	Farm, free-range	A/chicken/Bhutan/507/2012
7	01/27/2012	Chukha	Phuntsholing	Unknown	A/wild bird/Bhutan/325/2012
8	01/27/2012	Chukha	Phuntsholing	Unknown	A/wild bird/Bhutan/326/2012
9	01/27/2012	Chukha	Phuntsholing	Unknown	A/wild bird/Bhutan/327/2012
10	01/27/2012	Chukha	Phuntsholing	Unknown	A/wild bird/Bhutan/328/2012
11	01/27/2012	Chukha	Phuntsholing	Unknown	A/wild bird/Bhutan/329/2012
12	01/27/2012	Chukha	Phuntsholing	Farm, free-range	A/chicken/Bhutan/330/2012
13	01/27/2012	Chukha	Phuntsholing	Farm, free-range	A/chicken/Bhutan/331/2012
14	01/29/2012	Thimphu	Thimphu	Farm, free-range	A/chicken/Bhutan/345/2012
15	01/29/2012	Thimphu	Thimphu	Farm, free-range	A/chicken/Bhutan/346/2012
16	01/29/2012	Thimphu	Thimphu	Farm, free-range	A/chicken/Bhutan/347/2012
17	01/29/2012	Thimphu	Thimphu	Farm, free-range	A/chicken/Bhutan/349/2012
18	01/30/2012	Chukha	Wangdigatsel	Farm, free-range	A/chicken/Bhutan/352/2012
19	01/30/2012	Chukha	Wangdigatsel	Farm, free-range	A/wild bird/Bhutan/356/2012
20	01/30/2012	Chukha	Bjabchu	Farm, free-range	A/yellow billed duck/Bhutan/358/2012
21	01/30/2012	Chukha	Bjabchu	Farm, free-range	A/chicken/Bhutan/359/2012
22	01/31/2012	Thimphu	Thimphu	Unknown	A/wild bird/Bhutan/357/2012
23	02/11/2012	Thimphu	Thimphu	Farm, free-range	A/chicken/Bhutan/297/2012
24	02/11/2012	Thimphu	Thimphu	Farm, free-range	A/chicken/Bhutan/298/2012
25	02/14/2012	Thimphu	Thimphu	Farm, free-range	A/chicken/Bhutan/257/2012
26	02/14/2012	Thimphu	Thimphu	Farm, free-range	A/chicken/Bhutan/258/2012
27	02/14/2012	Thimphu	Thimphu	Farm, free-range	A/chicken/Bhutan/259/2012
28	02/14/2012	Thimphu	Thimphu	Farm, free-range	A/chicken/Bhutan/260/2012
29	02/14/2012	Chukha	Phuntsholing, Balujhora	Farm, free-range	A/chicken/Bhutan/265/2012
30	02/18/2012	Chukha	Gedu	Farm, free-range	A/chicken/Bhutan/413/2012
31	02/18/2012	Chukha	Gedu	Farm, free-range	A/chicken/Bhutan/414/2012
32	02/18/2012	Chukha	Gedu	Farm, free-range	A/chicken/Bhutan/415/2012
33	02/18/2012	Chukha	Gedu	Farm, free-range	A/chicken/Bhutan/416/2012
34	02/18/2012	Chukha	Gedu	Farm, free-range	A/chicken/Bhutan/417/2012
35	02/18/2012	Chukha	Gedu	Farm, free-range	A/chicken/Bhutan/418/2012
36	02/29/2012	Chukha	Gedu	Unknown	A/wild bird/Bhutan/308/2012
37	03/18/2012	Thimphu	Thimphu	Farm, free-range	A/chicken/Bhutan/1026/2012
38	03/18/2012	Thimphu	Thimphu	Farm, free-range	A/chicken/Bhutan/1028/2012
39	03/18/2012	Thimphu	Thimphu	Farm, free-range	A/chicken/Bhutan/1029/2012
40	03/18/2012	Thimphu	Thimphu	Farm, free-range	A/chicken/Bhutan/1030/2012
41	03/18/2012	Thimphu	Thimphu	Farm, free-range	A/chicken/Bhutan/1031/2012
42	09/10/2012	Chukha	Phuntsholing, Rinchening	Unknown	A/chicken/Bhutan/933/2012
43	09/10/2012	Chukha	Phuntsholing, Rinchening	Unknown	A/chicken/Bhutan/934/2012
44	10/11/2012	Chukha	Phuntsholing, Rinchening	Unknown	A/chicken/Bhutan/935/2012
45	10/11/2012	Chukha	Phuntsholing, Rinchening	Unknown	A/chicken/Bhutan/936/2012

Technical Appendix 1 Table 2. Amino acid differences in the genomes of A/chicken/Bangladesh/22478/2014 (pH5N1) and A/chicken/Bhutan/346/2012 (rH5N1)

Gene	Virus	Amino acid positions																										
		64	299	339	344	345	355	457	553	702																		
PB2	Ck/BD/22478	I	K	I	V	F	R	I	V	K																		
	Ck/Bh/346	M	R	T	L	L	K	V	I	R																		
PB1	Ck/BD/22478	11	12	14*	20	54	105	149	157	158†	191	215 †	261	384	386	398	423	429	456	515	578	584	621	635	644	667		
	Ck/Bh/346	R	V	V	T	E	T	I	A	N	V	R	S	I	K	D	I	K	R	A	R	H	Q	R	I	T		
PB1-F2	Ck/BD/22478	6	7	13	16	18	20	21	22	23	27	30	33	35	36	37	40	48	50	57	60	70	74	75	77	79	84	
	Ck/Bh/346	G	I	T	T	I	K	K	V	S	T	Q	P	S	T	R	D	P	G	Y	Q	E	L	R	L	R	N	
PA	Ck/BD/22478	20	57	59	61	118	231	405	425	614																		
	Ck/Bh/346	A	R	D	T	V	T	C	F	A																		
HA‡§	Ck/BD/22478	95	121	156	174	189	236	266	320	396	523																	
	Ck/Bh/346	L	F	T	I	R	K	R	N	V	A																	
NA¶	Ck/BD/22478	8	13	19	35	100	346	373	382	434																		
	Ck/Bh/346	I	V	T	G	H	V	I	D	G																		
M2	Ck/BD/22478	82																										
	Ck/Bh/346	N																										
NS1#	Ck/BD/22478	76	114																									
	Ck/Bh/346	G	P																									
NS2	Ck/BD/22478	26	48																									
	Ck/Bh/346	G	M																									

*Amino acid substitution V14A in the PB1 has been associated with reduced polymerase activity and reduced transmissibility in chickens in recombinant influenza A viruses with HA and NA from highly pathogenic H5N1 and internal genes from low pathogenicity viruses (Suzuki Y, Uchida Y, Tanikawa T, Maeda N, Takemae N, Saito T. Amino acid substitutions in PB1 of avian influenza viruses influence pathogenicity and transmissibility in chickens. J Virol. 2014;88:11130–9).

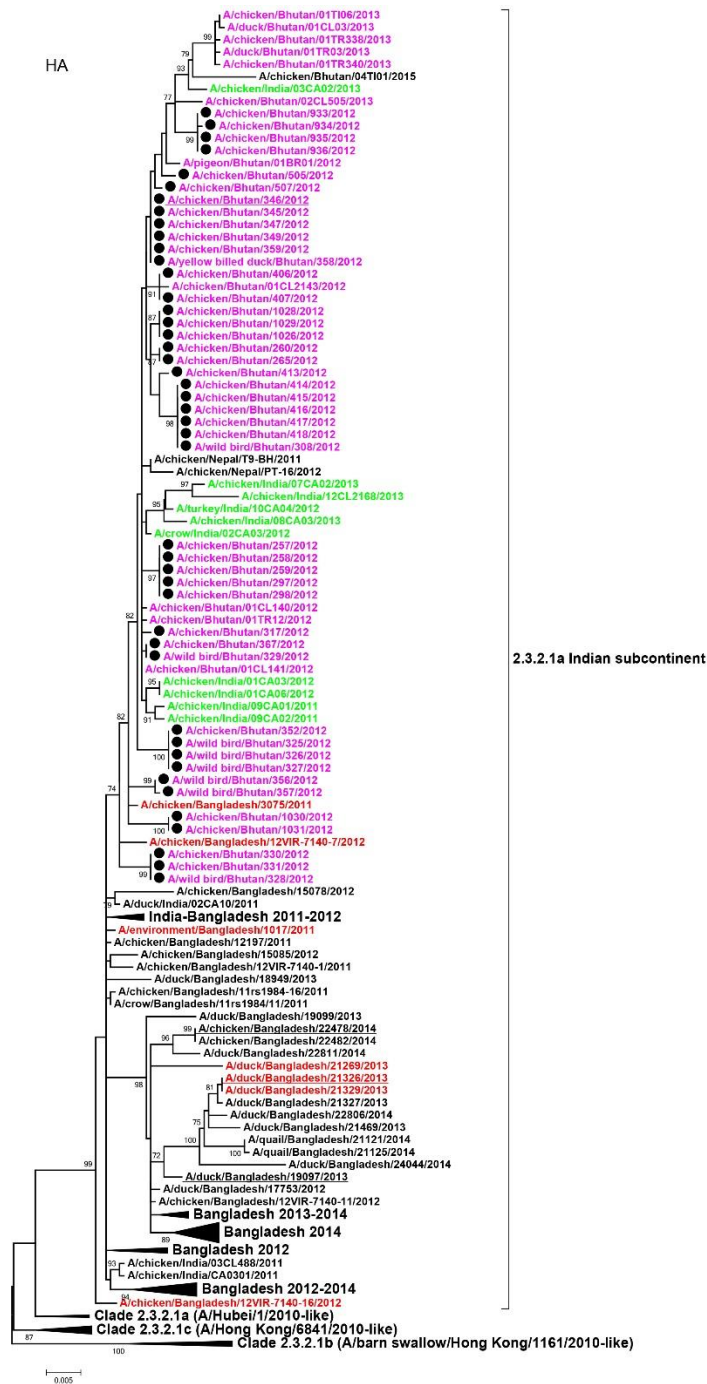
†N158S and R215K have been reported to potentially decrease the replication and pathogenicity of reassortant H5N1 viruses [Wasilenko JL, Lee CW, Sarmento L, Spackman E, Kapczynski DR, Suarez DL, et al. NP, PB1, and PB2 viral genes contribute to altered replication of H5N1 avian influenza viruses in chickens. J Virol. 2008;82(9):4544-53].

‡Mature H5 HA.

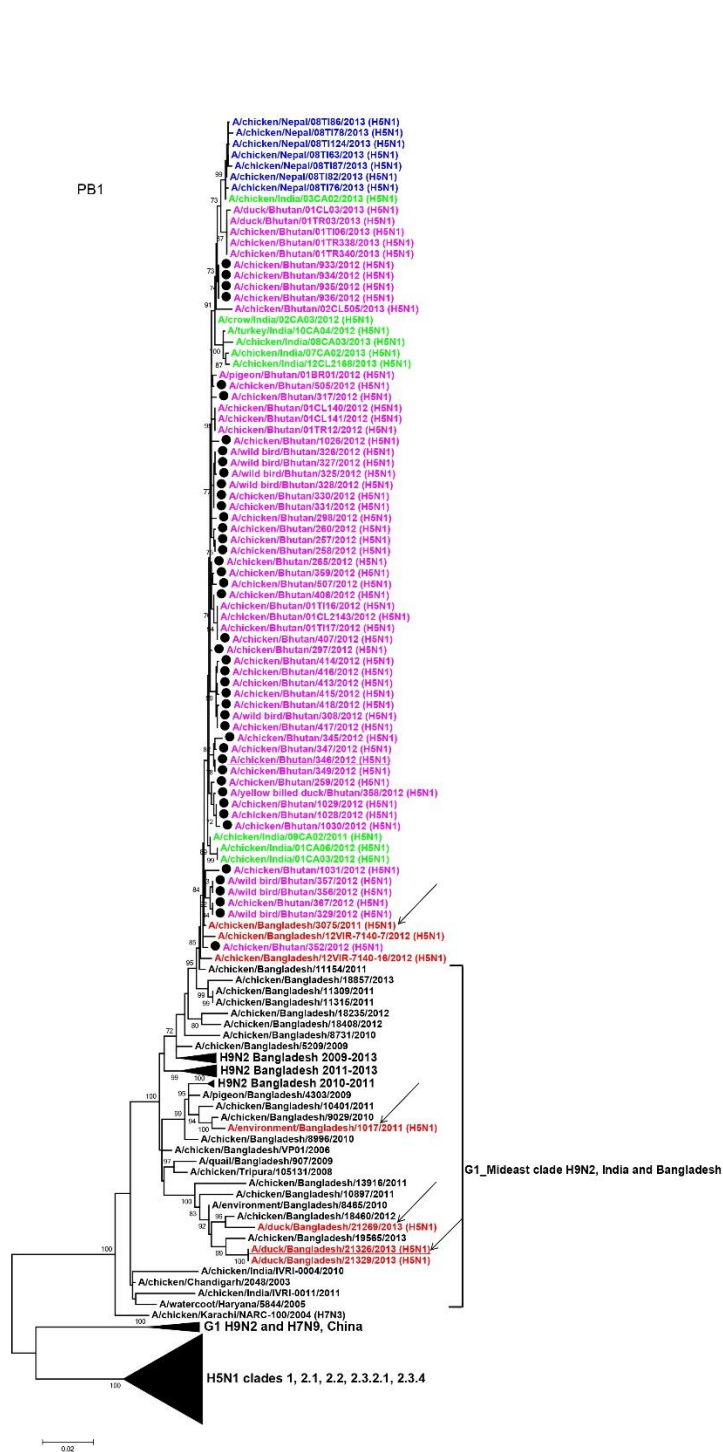
§The 2 viruses have identical polybasic cleavage-site motifs in the hemagglutinin (PQRERRRK_RGLF).

¶Both viruses have a 20-aa deletion at positions 49–68 of the neuraminidase.

#Both viruses have a 5-aa deletion at positions 80–84 of the NS1 gene.



Technical Appendix Figure 1. Phylogenetic relationships of the hemagglutinin gene of highly pathogenic avian influenza (HPAI) (H5N1) viruses isolated in Bhutan. Phylogeny was reconstructed by using the maximum likelihood method based on the Tamura-Nei model and with 1,000 bootstrap replicates. Numbers at the branches indicate bootstrap values; only values >70 are shown. HPAI H5N1 viruses from the reassortant genotype are color coded. Red, rH5N1 viruses isolated in Bangladesh; green, rH5N1 viruses isolated in India; pink, rH5N1 viruses isolated in Bhutan; black circle, viruses isolated during this study. Virus strains that are underlined were used for in vitro or in vivo experiments.



Technical Appendix Figure 2. Phylogenetic relationships of the PB1 gene of highly pathogenic influenza (HPAI) (H5N1) viruses isolated in Bhutan. Phylogeny was reconstructed by using the maximum likelihood method based on the Tamura-Nei model with 1,000 bootstrap replicates. Numbers at the branches indicate bootstrap values; only values >70 are shown. HPAI H5N viruses from the reassortant genotype are color coded. Red, rH5N1 viruses isolated in Bangladesh; green, rH5N1 viruses isolated in India; blue, rH5N1 viruses isolated in Nepal; pink, rH5N1 viruses isolated in Bhutan. Black circle shows viruses isolated during this study. Virus strains that are underlined were used for in vitro or in vivo experiments. Arrows show separate reassortment events.

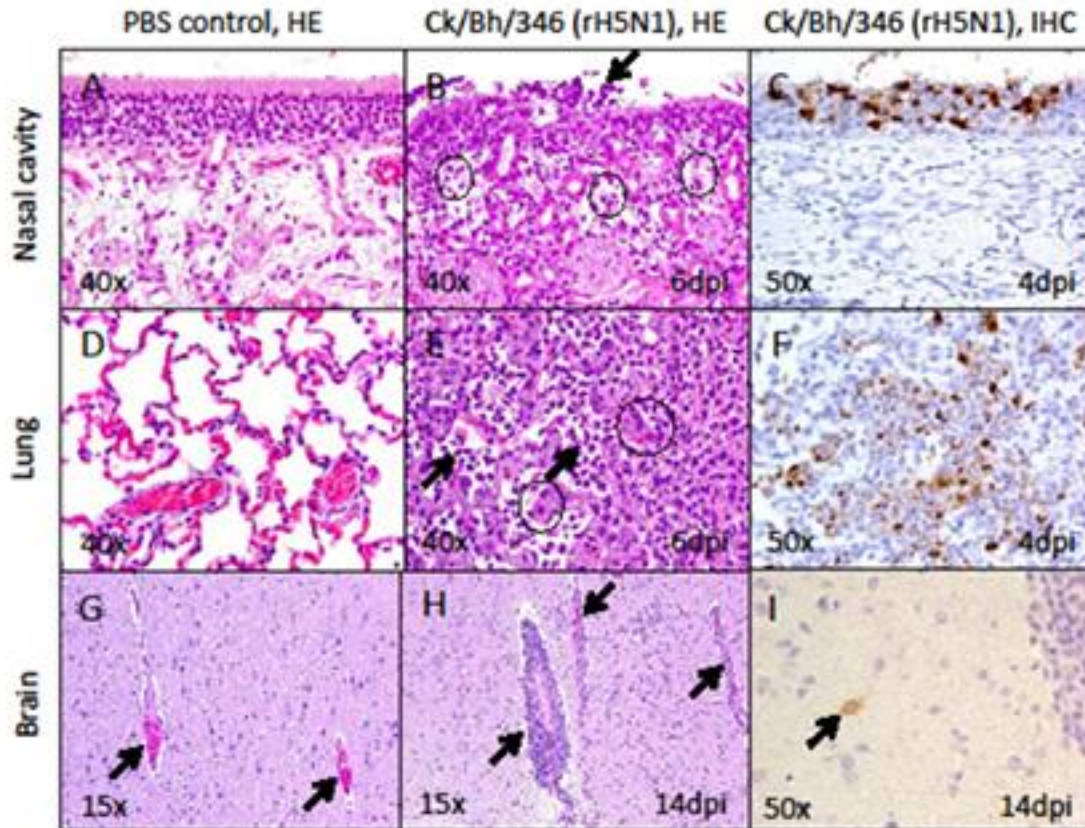
Highly Pathogenic Reassortant Avian Influenza A(H5N1) Virus Clade 2.3.2.1a in Poultry, Bhutan

Technical Appendix 2

Results of Histopathologic and Immunohistochemical Analyses

Histopathologic examination of ferrets inoculated intranasally with 10^6 EID₅₀/0.5 mL of Ck/Bh/346(rH5N1) revealed upper and lower respiratory tract involvement (Technical Appendix 2 Figure, center column). Inflammation, degeneration, edema, and necrosis of the respiratory and olfactory epithelium of the nasal cavity, along with generalized pneumonia involving bronchi, bronchioles, and alveoli, occurred at 4 and 6 dpi; however, at 14 dpi the number of lesions was substantially lower. A donor ferret that displayed lethargy between 3 and 10 dpi was euthanized at 14 dpi for tissue collection and was diagnosed post mortem with mild meningoencephalitis with multifocal lesions in the olfactory bulb (Technical Appendix 2 Figure). There were no pathologic changes in other tissues of this animal.

Immunohistochemical analysis showed multiple foci of influenza A virus nucleocapsid protein (NP)-positive epithelial cells in the nasal cavity and the alveolar tissue of the lungs of inoculated ferrets at 4 dpi (Figure, right column), but only scattered individual positive cells at 6 dpi. A single NP-positive cell was detected at 14 dpi in the brain of the ferret with meningoencephalitis (Figure, bottom right).



Technical Appendix Figure. Results of histopathologic examination and immunohistochemical analysis of ferret tissues after infection with *A/chicken/Bhutan/346/2012* (rH5N1). The images in the left column are tissue sections from a control ferret inoculated with PBS; the images in the middle and right columns are tissue sections from a ferret infected with *A/chicken/Bhutan/346/2012* (rH5N1). Magnifications are shown in the bottom left corner of each panel; times postinfection are indicated in the bottom right corner of panels showing infected ferret tissues. A) Normal septal olfactory epithelium and submucosal glands in the nasal cavity of an infected ferret. B) Septal olfactory epithelial necrosis, sloughing (arrow), submucosal inflammation (inflammatory cells are circled), and edema in the nasal cavity of an infected ferret. C) Immunohistochemical analysis of septal olfactory epithelium of an infected ferret. Cells containing the influenza A NP are stained brown. D) Normal alveolar tissue with thin alveolar walls and blood vessels not surrounded by inflammatory cells. E) Alveolitis (inflammation of the alveolar tissue) in an infected ferret, showing septal thickening, hyperplasia, hypertrophy of the pneumocytes (circled), and the presence of large numbers of inflammatory cells (arrows). F) Immunohistochemical analysis of an area of alveolitis in the lung of an infected ferret. Cells containing the influenza A NP are stained brown. G) Normal brain tissue with blood vessels (arrows). H) Encephalitis (inflammation of the brain) in an infected ferret, showing perivascular cuffing (arrows) at the level of the optic chiasma. Note the presence of large numbers of mononuclear inflammatory cells and neutrophils. I) The arrow shows a single influenza A–infected cell (the viral NP is stained brown) in an area of encephalitis in the brain of an

infected ferret. Perivascular cuffing is visible in the upper right corner of the image. rH5N1, reassortant H5N1; HE, hematoxylin-and-eosin staining; NP, nucleocapsid protein; IHC, immunohistochemical staining.