Supporting Information

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SI Materials and Methods

Infection and Transmission in Ferrets. Infection and transmission were carried out as described previously (1). Briefly, 3- to 7-mo-old ferrets were purchased from Triple F Farms. Ferrets were housed in BSL2 animal rooms for 1 wk to monitor general health and establish baseline body temperature and weight. Before infection, ferrets were bled for serum collection and had an implantable transponder (Bio Medic Data Systems Inc.) placed subcutaneously for body temperature recording and identification. Ferrets that were seronegative by hemagglutination inhibition assay to seasonal H3N2, seasonal H1N1, H9N2, and pH1N1 were used for these studies. Infection and transmission studies were performed in an ABSL3$^+$ animal facility in wire cages inside HEPA filtered isolators, as previously described (1). Animal studies were conducted under guidelines approved by the Animal Care and Use Committee of the University of Maryland and the Center for Disease Control and Prevention (protocol number RO-09-93). Each experiment consisted of three ferrets in duplicate for each virus. One ferret was inoculated intranasally with 10^6 tissue culture infectious dose ($TCID_{50}$) of virus in phosphate buffered solution (PBS) after light anesthesia with ketamine (20 mg/kg) and xylazine (1 mg/kg) administered intramuscularly. At 1 d postinoculation (dpi), two naive ferrets were added to the cage. One ferret was added in direct contact with the inoculated ferret, but the second naive ferret was added to the other half of the cage separated by two layers of thin wire mesh, allowing no physical contact. Body weight and temperature were measured daily and nasal washes were collected for 14 dpi. Nasal washes were collected by anesthetizing the ferrets as described above and inducing sneezing with 1 mL of PBS collected in a Petri dish. Washes were collected and brought up to 1-mL volume and tested for virus by Flu Detect Antigen Capture Test Strip (Synbiotics Corp.). Additional aliquots were stored at −80 °C for titering and sequencing. Blood was collected at 14 dpi for serum collection. Two additional ferrets were infected with each virus for pathology and virus localization at 3 and 5 dpi.

Virus Localization. Virus was located and visualized in ferret tissue by standard immunohistochemistry methods. Briefly, slides were deparaffinized by two 3-min xylene washes followed by 2 min in ethanol at descending concentrations, from 100%, 90%, 80%, to 70% and rinsed in phosphate buffed solution (PBS Sigma). Endogenous peroxidases were blocked by 15-min incubation in 2% H_2O_2 in methanol at 4 °C. Tissues were blocked overnight at 4 °C with 1% BSA in PBS. The following day, tissues were immersed in anti-NP monoclonal antibodies that were diluted 1:500 in PBS for 1 h at room temperature. Following three 5-min rinses in PBS, tissues were incubated with anti-mouse antibody diluted 1:1,000 in PBS. Three more 5-min PBS rinses ensued and then tissues were covered with aminoethylcarbazole (DAKO) for 10 min and rinsed in distilled water. After a 30-min incubation in hematoxylin and rinse in tap water, coverslips were mounted and tissue was visualized at 200× magnification. Three slides were analyzed per ferret, per tissue.

Blocking ELISA. In the blocking ELISA, A/Guinea fowl/Hongkong/WF10(H9N2) (WF10) virus was used as a coating antigen and HRP-conjugated monoclonal antibody against AIV-H9-HA-3G8 was selected the as detection antibody. In brief, 96-well plates were coated in WF10 diluted in carbonate/bicarbonate buffer (pH 9.6) for 12 h at 4 °C. After blocking, the plates with 5% (wt/vol) nonfat milk in PBS for 1 h at 37 °C, the serum samples were diluted 1:4 in dilution buffer (0.5% BSA in PBS) and added to the wells (100 μL per well) and the mixture was incubated at 37 °C for 1 h. After washing once, 100 μL HRP-conjugated 3G8 antibodies (0.1 ng/mL) in dilution buffer was added to well and the mixture was incubated for 1 h at 37 °C. After five washes, the development was performed using the TMB substrate system (KPL) for 10 min. The ratio of the OD630 value of the sample wells (S) to that of the negative control wells (N) was calculated, and S/N values less than 0.5 or 0.6 were considered as positive in the ELISA.

Serum collected from each ferret at the end of the experiments was tested for seroconversion by hemagglutination inhibition assay and by a blocking ELISA. Every ferret that had detectable levels of shed virus proved positive by both hemagglutination inhibition and the blocking ELISA, with the exception of the RC2WF10 ferret with delayed infection. This ferret was killed only 6 d after first shedding virus and thus did not seroconvert. Additionally, the two RC1WF10 ferrets that never shed virus never seroconverted.

1. Wan H, et al. (2008) Replication and transmission of H9N2 influenza viruses in ferrets: Evaluation of pandemic potential. *PLoS ONE* 3:e2923.

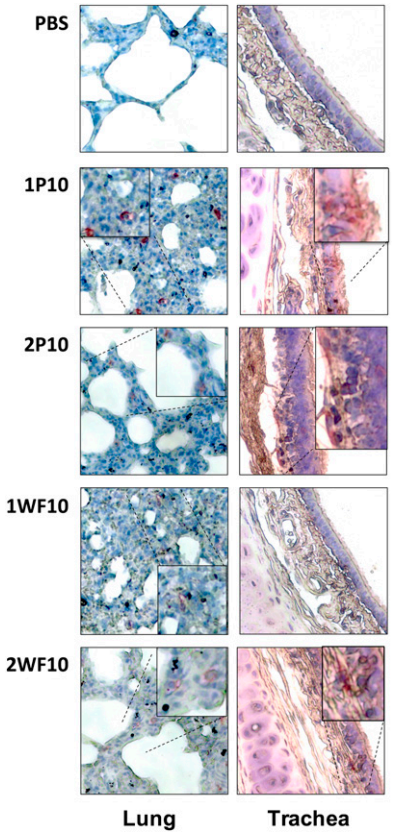

Fig. S1. Virus localization in infected tissue. Unstained tissue from ferrets after 5 dpi with 1×10^6 $TCID_{50}$ of either 1P10, 2P10, 1WF10, or 2WF10 underwent anti-NP–based immunohistochemistry, as described in *Materials and Methods*. Blue/purple represents nuclear staining. Red marks area positive for NP protein. Insets show selected areas with higher magnification for visualization purposes. (Magnification: 200×.)

Table S1. Ferrets showed moderate weight loss following infection with reassortant H9:pH1N1 viruses

Virus	Group	Body weight loss	Serum (HI titer)	Blocking ELISA
1P10	DI	5.976 ± 0.592	2,560, 2,560	+, +
	DC	5.877 ± 2.654	640, 640	+, +
	RC	6.041 ± 1.652	2,560, 1,280	+, +
2P10	DI	8.279 ± 0.902	2,560, 2,560	+, +
	DC	2.947 ± 0.336	1,280, 640	+, +
	RC	6.497 ± 2.523	320, 320	+, +
1WF10	DI	3.708 ± 0.479	1,280, 1,280	+, +
	DC	6.34 ± 6.788	2,560, 1,280	+, +
	RC	–	<10	–, –
2WF10	DI	4.298 ± 2.388	1,280, 1,280	+, +
	DC	5.406 ± 0.856	640, 320	+, +
	RC	4.647 ± 2.796	<10, 1,280	–, +

Direct inoculated (DI), direct contact (DC), and respiratory contact (RC) ferrets peak percent body weight loss during infection. The "–" in the Body weight loss column indicates no weight loss observed. HI, hemagglutination inhibition. In the Blocking ELISA column, "+" indicates positive and "–" indicates negative for anti H9 HA antibodies.