Supplementary materials

Advancing A(H5N1) influenza risk assessment in ferrets through comparative evaluation of airborne virus shedding patterns.

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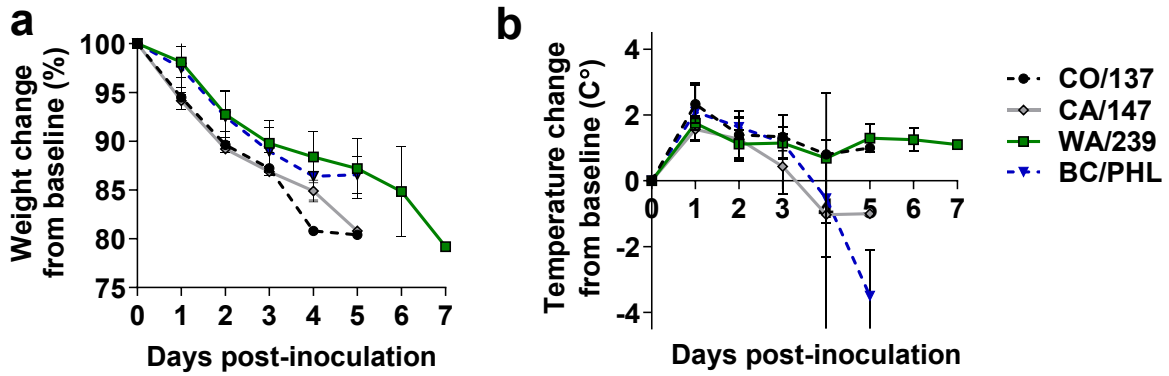
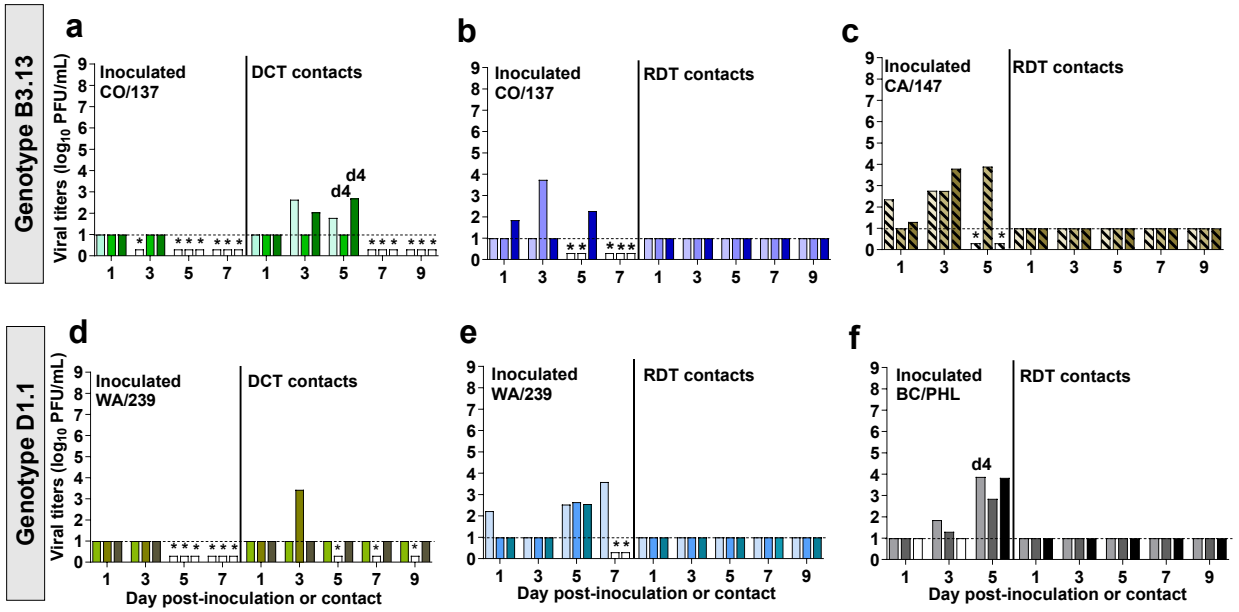
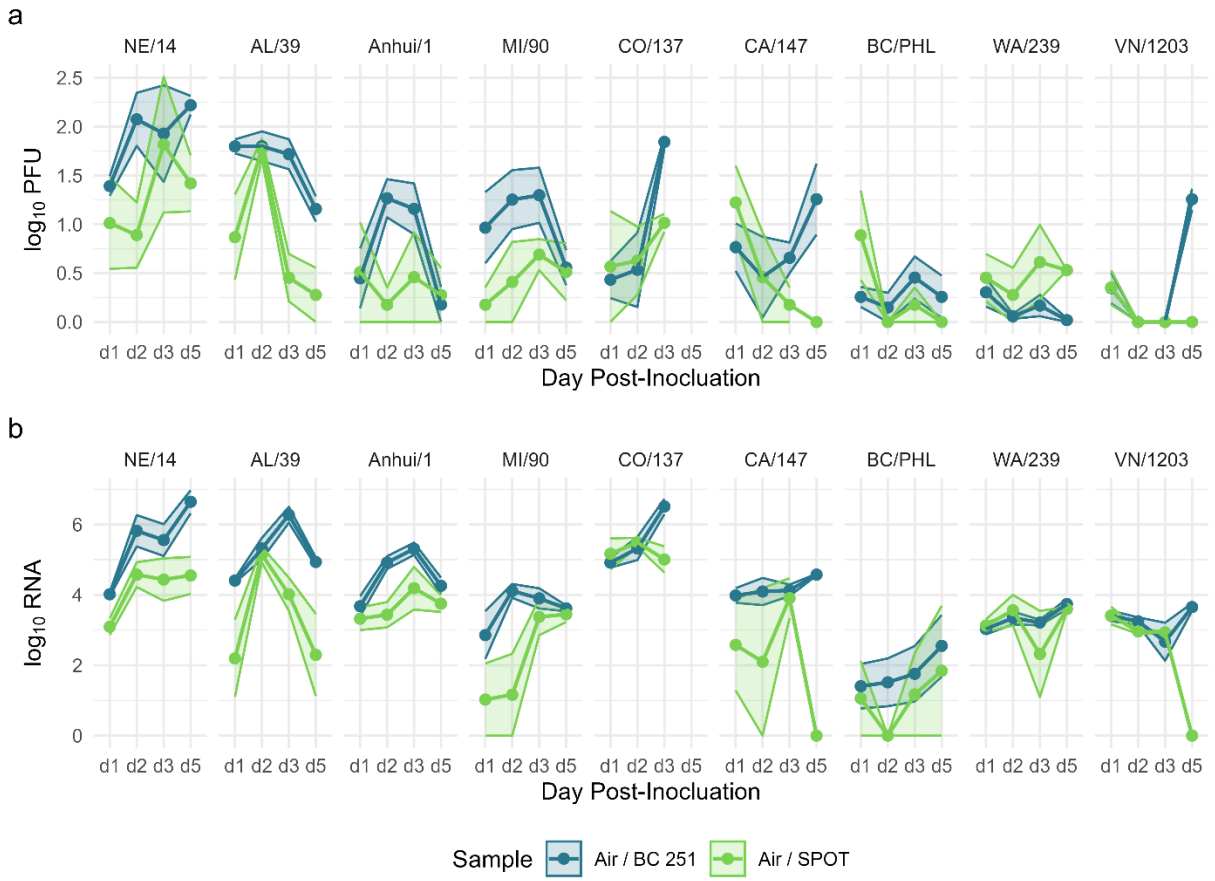


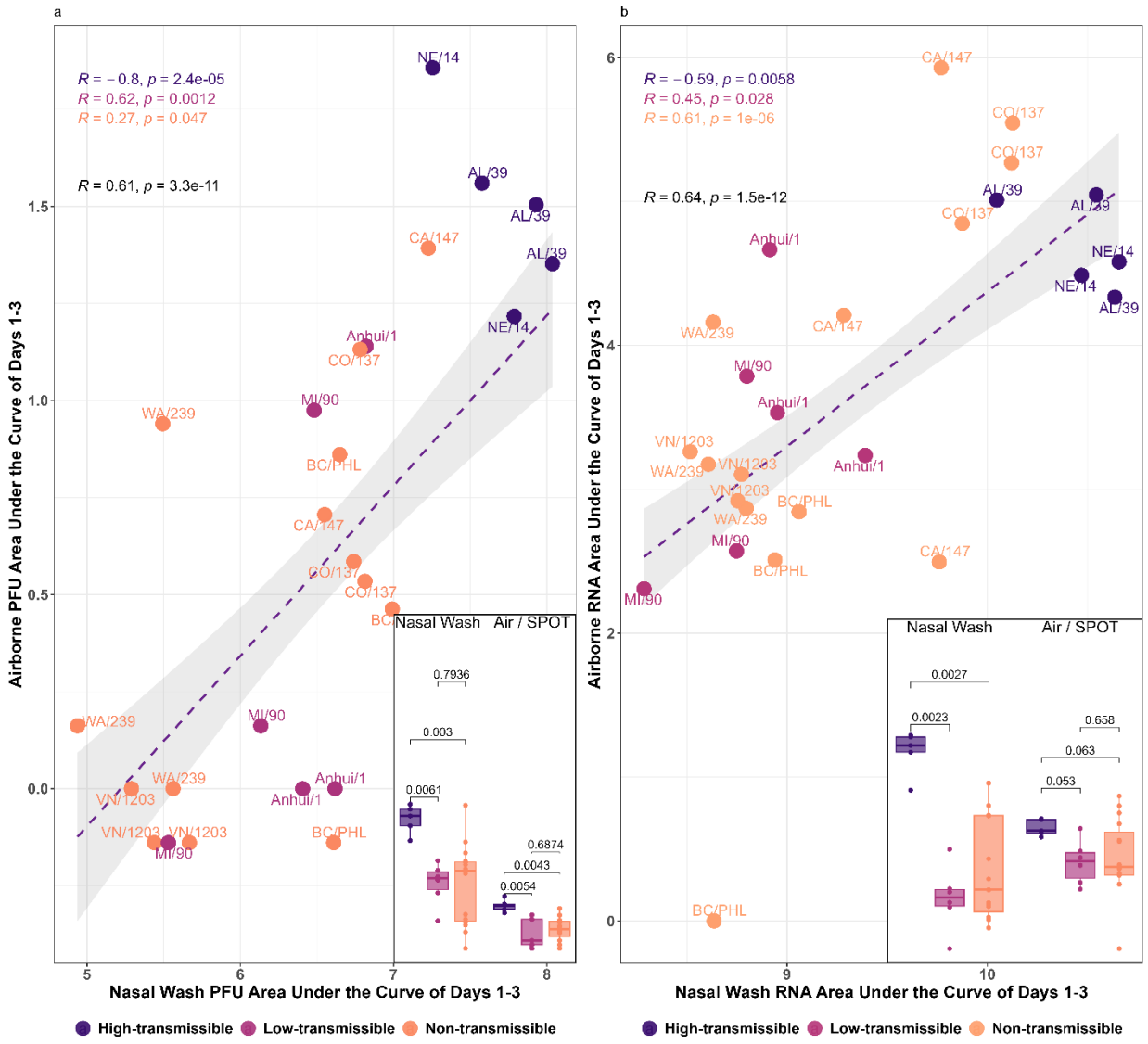
Supplementary Fig. 1. Weight and temperature changes in ferrets inoculated with genotype B3.13 and D1.1 A(H5N1) viruses. Ferrets were inoculated with 6 $\log_{10}$ PFU of A/Colorado/137/2024 (CO/137) (n=6), A/California/147/2024 (CA/147) (n=3), A/Washington/239/2024 (WA/239) (n=6), and A/British Columbia/PHL-2032/2024 (BC/PHL) (n=3) viruses and weight (a) and temperature (b) changes from baseline were assessed daily until study endpoint. Error bars represent the standard deviation. The graphs were generated using GraphPad Prism and the source data has been deposited on GitHub[1].



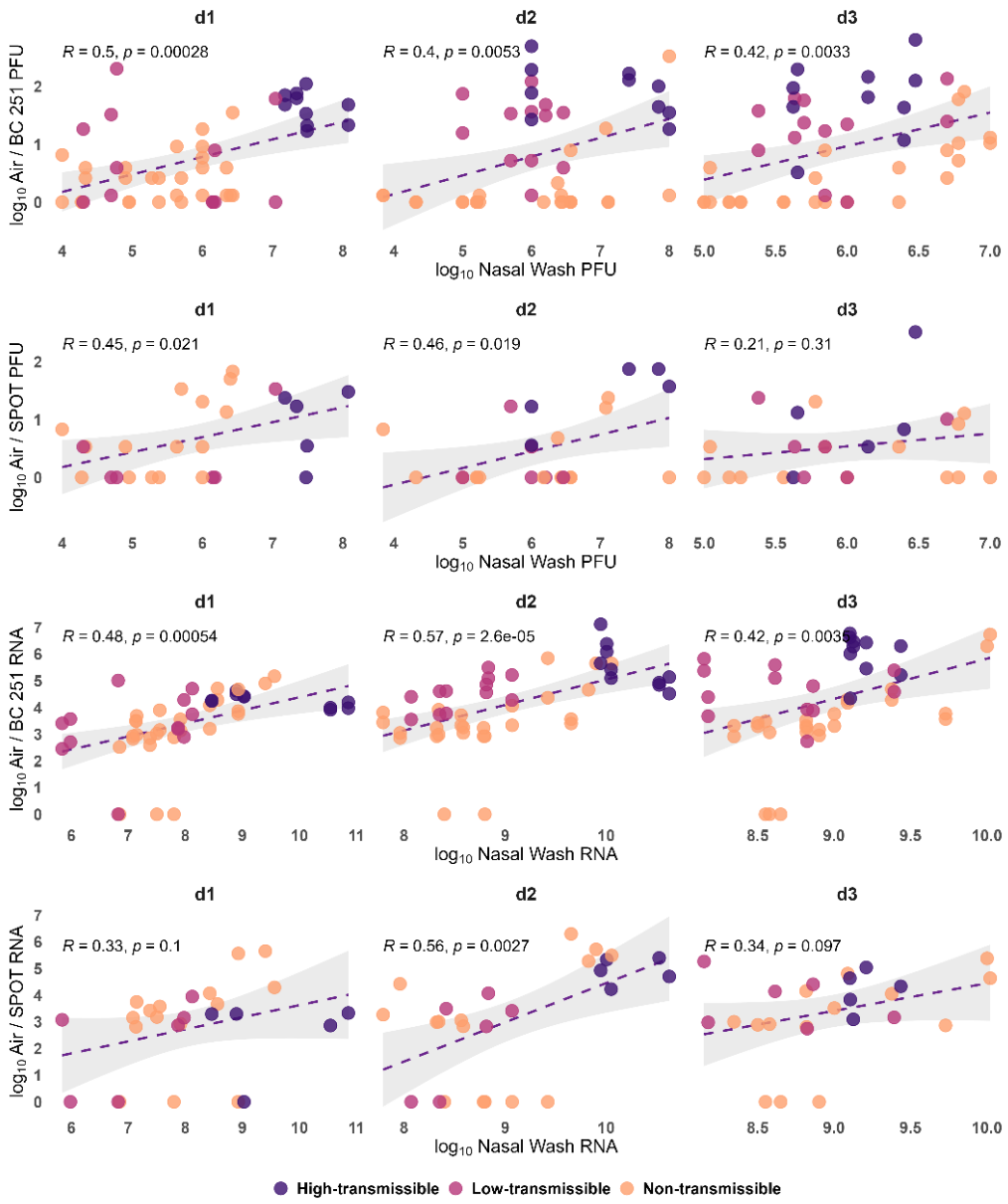
Supplementary Fig. 2. Detection of genotype B3.13 and D1.1 A(H5N1) viruses in rectal swabs of inoculated and contact ferrets. Ferrets were inoculated with 6 log10 PFU of (a, b) A/Colorado/137/2024 (CO/137), (c) A/California/147/2024 (CA/147), (d, e) A/Washington/239/2024 (WA/239), (f) and A/British Columbia/PHL-2032/2024 (BC/PHL) viruses. Transmission to naïve contacts was tested using the direct contact transmission model (DCT) and respiratory droplet transmission model (RDT). Bars on the left represent inoculated ferrets (n=3 per group), and the bars on the right represent contact ferrets (n=3 per group). Bars represent titers in rectal swabs for individual animals collected every other day post-inoculation or contact, or on the day of euthanasia (day 4 indicated on top of the bar). White bars with asterisks denote animals that succumbed to infection prior to sample collection. Limit of detection: 10 PFU/mL. The graphs were generated using GraphPad Prism and the source data has been deposited on GitHub[1].



Supplementary Fig. 3. Detection of airborne influenza virus using BC251 and SPOT samplers. Air samples were collected from ferrets inoculated with A/Nebraska/14/2019 A(H1N1) (NE/14; n=2), A(H9N2) A/Anhui-Lujiang/39/2018 (AL/39; n=3), A(H7N9) A/Anhui/1/2013 (Anhui/1; n=3), A(H5N1) A/Michigan/90/2024 (MI/90; n=3), A(H5N1) A/Colorado/137/2024 (CO/137; n=3), A(H5N1) A/California/147/2024 (CA/147; n=3), A(H5N1) A/Washington/239/2024 (WA/239; n=3), A(H5N1) A/British Columbia/PHL-2032/2024 (BC/PHL; n=3), and A(H5N1) A/Vietnam/1203/2004 (VN/1203; n=3) using BC251 (teal) and SPOT samplers (green). Virus titers in each sample were evaluated for **(a)** infectious virus and **(b)** genomic RNA copy load. Data are presented as $\log_{10}$ mean PFU or RNA copies per 105 liters of air. Shaded areas and error bars represent the standard error of the mean, calculated from all replicates, and are shown for descriptive purposes only to help visualize data variability for each sample type and are not used to draw statistical conclusions. The graphs were generated using R and the source data and code has been deposited on GitHub[1].



Supplementary Fig. 4. Correlation between viral loads in nasal washes and air samples from inoculated ferrets using SPOT sampler. Area under the curve (AUC) values were calculated using infectious virus (PFU) **(a)** and viral RNA copy **(b)** titers from nasal wash and air samples collected on days 1, 2, and 3 post-inoculation with 6 $\log_{10}$ PFU of A(H1N1)pdm09 A/Nebraska/14/2019 (NE/14) (n=2), A(H9N2) A/Anhui-Lujiang/39/2018 (AL/39) (n=3), A(H7N9) A/Anhui/1/2013 (Anhui/1) (n=3), A(H5N1) A/Michigan/90/2024 (MI/90) (n=3), A(H5N1) A/Colorado/137/2024 (CO/137) (n=3), A(H5N1) A/California/147/2024 (CA/147) (n=3), A(H5N1) A/British Columbia/PHL-2032/2024 (BC/PHL) (n=3), A(H5N1) A/Washington/239/2024 (WA/239) (n=3), and A(H5N1) A/Vietnam/1203/2004 (VN/1203) (n=3). Viruses were grouped based on their known airborne transmissibility profiles in the ferret model into three categories: high-transmissible (NE/14 and AL/39; 100-83% transmission frequency; purple circles and bars; n=5), low-transmissible (MI/90 and Anhui/1; 50-33% transmission frequency; magenta circles and bars; n=6), and non-transmissible (CO/137, CA/147, BC/PHL, WA/239, VN/1203; 0% transmission frequency; orange circles and bars; n=15). Correlations between AUC values were analyzed using Pearson's method, with 95% confidence intervals shown. Pairwise comparisons were conducted using the Kruskal-Wallis test with Dunn's multiple comparisons post hoc-analysis. The graphs were generated using R and the source data and code has been deposited on GitHub[1].



Supplementary Fig. 5. Correlation between virus load in nasal washes and air samples from inoculated ferrets for each day of collection. Viruses were grouped based on their known airborne transmissibility profiles into three categories: high-transmissible (A/Nebraska/14/2019 (NE/14) and A/Anhui-Lujiang/39/2018 (AL/39); 100-83% transmission frequency; purple circles), low-transmissible (A/Michigan/90/2024 (MI/90) and A/Anhui/1/2013(Anhui/1); 50-33% transmission frequency; magenta circles), and non-transmissible (A/Colorado/137/2024 (CO/137), A/California/147/2024 (CA/147), A/Washington/239/2024 (WA/239), A/British Columbia/PHL-2032/2024 (BC/PHL) and A/Vietnam/1203/2004 (VN/1203); 0% transmission frequency; orange circles). Correlations between nasal wash titers and SPOT or BC251 air samples were analyzed for each day of collection (days 1, 2, and 3 post-inoculation) using Pearson's method, with 95% confidence intervals shown. PFU represents infectious virus, and RNA represents viral genomic RNA copies. The graphs were generated using R and the source data and code has been deposited on GitHub[1].

Supplementary Table 1. Average viral titers in nasal wash and air samples collected from inoculated ferrets.

Virus[a]	Day p.i.[b]	Sample type	Air collection time (min) [c]	Number of replicates	log_{10} RNA copies/mL of NW or 105L of air[d]			log_{10} PFU/mL of NW or 105L of air[d]		
					Mean	Standard error	Standard deviation	Mean	Standard error	Standard deviation
NE/14 (n=2)	d1	Nasal Wash	NA	2	10.70	0.16	0.22	7.79	0.29	0.42
		Air/BC 251	30	2	4.02	0.06	0.12	1.39	0.10	0.20
		Air/BC 251	60	2						
		Air/SPOT	30	2	3.09	0.23	0.33	1.01	0.47	0.66
	d2	Nasal Wash	NA	2	10.00	0.05	0.07	6.00	0.00	0.00
		Air/BC 251	30	2	5.82	0.45	0.89	2.07	0.27	0.54
		Air/BC 251	60	2						
		Air/SPOT	30	2	4.57	0.35	0.50	0.89	0.33	0.47
	d3	Nasal Wash	NA	2	9.16	0.05	0.07	6.07	0.41	0.58
		Air/BC 251	30	2	5.56	0.45	0.90	1.93	0.49	0.99
		Air/BC 251	60	2						
		Air/SPOT	30	2	4.44	0.60	0.85	1.82	0.70	0.98
	d5	Nasal Wash	NA	2	8.85	0.06	0.09	5.17	0.09	0.13
		Air/BC 251	30	2	6.64	0.33	0.47	2.22	0.10	0.13
		Air/SPOT	30	2	4.55	0.53	0.75	1.42	0.29	0.41
AL/39 (n=3)	d1	Nasal Wash	NA	3	8.80	0.17	0.30	7.33	0.09	0.15
		Air/BC 251	30	6*	4.41	0.06	0.15	1.80	0.07	0.18
		Air/SPOT	30	3	2.19	1.10	1.90	0.87	0.44	0.76
	d2	Nasal Wash	NA	3	10.39	0.19	0.33	7.75	0.18	0.30
		Air/BC 251	30	6*	5.31	0.30	0.74	1.80	0.15	0.37
		Air/SPOT	30	3	5.14	0.22	0.39	1.77	0.10	0.17
	d3	Nasal Wash	NA	3	9.22	0.11	0.19	6.06	0.23	0.40
		Air/BC 251	30	6*	6.28	0.23	0.55	1.72	0.15	0.38
		Air/SPOT	30	3	4.02	0.47	0.82	0.45	0.24	0.42
	d4	Nasal Wash	NA	3	8.12	0.24	0.42	5.68	0.16	0.28
		Air/BC 251	30	6*	4.93	0.12	0.30	1.16	0.13	0.32
		Air/SPOT	30	3	2.29	1.17	2.02	0.28	0.28	0.48
Anhui/1 (n=3)	d1	Nasal Wash	NA	3	7.99	0.07	0.12	6.45	0.29	0.51
		Air/BC 251	30	6*	3.68	0.29	0.70	0.45	0.31	0.75
		Air/SPOT	30	3	3.32	0.32	0.56	0.51	0.51	0.88
	d2	Nasal Wash	NA	3	8.91	0.08	0.14	6.22	0.13	0.23
		Air/BC 251	30	6*	4.92	0.18	0.44	1.27	0.20	0.48
		Air/SPOT	30	3	3.44	0.36	0.62	0.18	0.18	0.31
	d3	Nasal Wash	NA	3	8.72	0.36	0.63	5.69	0.18	0.31
		Air/BC 251	30	6*	5.31	0.17	0.43	1.16	0.26	0.64
		Air/SPOT	30	3	4.19	0.61	1.05	0.46	0.46	0.79
	d5	Nasal Wash	NA	3	9.04	0.10	0.17	5.12	0.06	0.11
		Air/BC 251	30	6*	4.26	0.23	0.57	0.18	0.18	0.44
		Air/SPOT	30	3	3.75	0.24	0.41	0.28	0.28	0.48
MI/90 (n=3)	d1	Nasal Wash	NA	3	6.22	0.31	0.53	4.59	0.15	0.26
		Air/BC 251	30	6*	2.86	0.68	1.66	0.96	0.36	0.89
		Air/SPOT	30	3	1.02	1.02	1.77	0.18	0.18	0.31
	d2	Nasal Wash	NA	3	8.28	0.11	0.18	5.57	0.30	0.51
		Air/BC 251	30	6*	4.11	0.20	0.48	1.25	0.30	0.74
		Air/SPOT	30	3	1.16	1.16	2.02	0.41	0.41	0.71
	d3	Nasal Wash	NA	3	8.62	0.22	0.39	6.06	0.33	0.56
		Air/BC 251	30	6*	3.90	0.29	0.70	1.30	0.28	0.69
		Air/SPOT	30	3	3.37	0.52	0.90	0.69	0.16	0.28
	d5	Nasal Wash	NA	3	8.11	0.20	0.35	5.26	0.29	0.51
		Air/BC 251	30	6*	3.62	0.08	0.20	0.56	0.18	0.44
		Air/SPOT	30	3	3.45	0.22	0.38	0.51	0.29	0.50
CO/137 (n=3)	d1	Nasal Wash	NA	3	9.30	0.19	0.33	5.89	0.33	0.57
		Air/BC 251	60	3	4.92	0.14	0.25	0.43	0.19	0.33
		Air/SPOT	60	3	5.17	0.44	0.76	0.57	0.57	0.98
	d2	Nasal Wash	NA	3	9.93	0.07	0.12	6.55	0.27	0.47
		Air/BC 251	60	3	5.32	0.33	0.57	0.53	0.38	0.66
		Air/SPOT	60	3	5.49	0.13	0.22	0.63	0.35	0.60
	d3	Nasal Wash	NA	2[#]	10.01	0.01	0.01	6.80	0.02	0.03
		Air/BC 251	60	2[#]	6.51	0.22	0.31	1.84	0.06	0.09
		Air/SPOT	60	2[#]	5.01	0.37	0.53	1.01	0.09	0.13
CA/147 (n=3)	d1	Nasal Wash	NA	3	8.64	0.15	0.26	6.14	0.14	0.25
		Air/BC 251	30	6*	3.98	0.21	0.51	0.77	0.24	0.59

		Air/SPOT	30	3	2.58	1.29	2.24	1.22	0.38	0.65
	d2	Nasal Wash	NA	3	9.38	0.17	0.29	7.18	0.45	0.79
		Air/BC 251	30	6*	4.09	0.39	0.95	0.46	0.41	1.01
		Air/SPOT	30	3	2.10	2.10	3.63	0.46	0.46	0.79
	d3	Nasal Wash	NA	3	9.40	0.19	0.32	6.44	0.30	0.52
		Air/BC 251	30	6	4.13	0.16	0.40	0.66	0.16	0.38
		Air/SPOT	30	3	3.91	0.57	0.98	0.18	0.18	0.31
	d5	Nasal Wash	NA	1	9.26	NA	NA	6.58	NA	NA
		Air/BC 251	30	2	4.57	0.02	0.03	1.26	0.36	0.51
		Air/SPOT	30	1	0.00	NA	NA	0.00	NA	NA
BC/PHL (n=3)	d1	Nasal Wash	NA	3	7.38	0.28	0.49	5.81	0.28	0.49
		Air/BC 255	30	6*	1.40	0.63	1.55	0.26	0.10	0.25
		Air/SPOT	30	3	1.06	1.06	1.83	0.89	0.46	0.79
	d2	Nasal Wash	NA	3	8.66	0.13	0.23	6.53	0.04	0.07
		Air/BC 256	30	6*	1.51	0.68	1.66	0.15	0.15	0.36
		Air/SPOT	30	3	0.00	NA	NA	0.00	NA	NA
	d3	Nasal Wash	NA	3	8.74	0.14	0.24	6.45	0.29	0.51
		Air/BC 257	30	6*	1.76	0.79	1.93	0.45	0.22	0.53
		Air/SPOT	30	3	1.17	1.17	2.03	0.18	0.18	0.31
	d5	Nasal Wash	NA	2	8.50	0.19	0.27	6.22	0.22	0.31
		Air/BC 252	30	4*	2.55	0.88	1.76	0.26	0.22	0.43
		Air/SPOT	30	2	1.84	1.84	2.61	0.00	NA	NA
WA/239 (n=3)	d1	Nasal Wash	NA	3	7.20	0.09	0.16	4.20	0.10	0.18
		Air/BC 253	30	6*	3.03	0.15	0.38	0.30	0.15	0.36
		Air/SPOT	30	3	3.13	0.18	0.30	0.45	0.24	0.42
	d2	Nasal Wash	NA	3	8.03	0.16	0.28	4.47	0.41	0.71
		Air/BC 254	30	6*	3.34	0.18	0.45	0.06	0.03	0.06
		Air/SPOT	30	3	3.56	0.44	0.75	0.28	0.28	0.48
	d3	Nasal Wash	NA	3	8.85	0.03	0.05	5.46	0.22	0.38
		Air/BC 255	30	6*	3.21	0.08	0.20	0.17	0.11	0.27
		Air/SPOT	30	3	2.32	1.22	2.12	0.61	0.38	0.66
	d5	Nasal Wash	NA	3	8.28	0.45	0.78	4.55	0.33	0.56
		Air/BC 256	30	6*	3.74	0.15	0.36	0.02	0.02	0.05
		Air/SPOT	30	3	3.60	0.06	0.11	0.53	NA	NA
VN/1203 (n=3)	d1	Nasal Wash	NA	3	7.54	0.22	0.38	5.16	0.24	0.41
		Air/BC 257	30	6*	3.40	0.15	0.36	0.35	0.16	0.38
		Air/SPOT	30	3	3.41	0.25	0.43	0.35	0.18	0.31
	d2	Nasal Wash	NA	3	8.49	0.08	0.15	5.14	0.07	0.12
		Air/BC 252	30	6*	3.25	0.11	0.27	0.00	NA	NA
		Air/SPOT	30	3	2.96	0.07	0.12	0.00	NA	NA
	d3	Nasal Wash	NA	3	8.47	0.07	0.12	5.14	0.08	0.13
		Air/BC 253	30	6*	2.67	0.54	1.32	0.00	NA	NA
		Air/SPOT	30	3	2.93	0.03	0.06	0.00	NA	NA
	d5	Nasal Wash	NA	3	8.65	0.13	0.23	5.57	0.19	0.32
		Air/BC 254	30	6*	3.65	0.11	0.27	1.26	0.11	0.27
		Air/SPOT	30	3	0	NA	NA	0	NA	NA

[a] Virus used for inoculation; number of inoculated ferrets is in parentheses.
[b] Day post inoculation
[c] Air samples were collected for 30 or 60 minutes.
[d] For consistency, all airborne virus titers are presented per 105 L of air to normalize for differences in collection times and flow rates between the samplers (BC 251: 3.5 L/min; SPOT: 1.5 L/min).
[*] One sample was collected from each ferret unless specified by the asterisk which indicates a duplicate sample were collected from each ferret.
[#] indicates reduced number of ferrets due to euthanasia prior to sample collection.
NA - not applicable
Standard error and standard deviation calculations for sample sizes of n=2 are shown for descriptive purposes only.

Supplementary Table 2. Variable odds ratios for BC251 compared to SPOT detection frequencies by Fisher's Exact Test.

Measurement	Air Sampler	Number of samples with virus detected	Number of samples with virus not detected	Total number of samples analyzed	% Detected	Odds Ratio	95% CI low	95% CI high	p-value
Infectious virus (PFU)	BC251	126	58	184	68.47826087	2.03668	1.192621	3.488985	0.006451
	SPOT	50	47	97	51.54639175				
Genomic RNA copies	BC251	172	12	184	93.47826087	4.180423	1.868857	9.784907	0.0001695
	SPOT	75	22	97	77.31958763				

Two-sided Fisher's Exact Test was used to calculate odds ratios with 95% confidence intervals, $p < 0.05$ is considered statistically significant.

Supplementary Table 3. Pairwise comparisons between viral loads in samples collected from ferrets inoculated with viruses displaying different airborne transmissibility profiles.

Sample	Variable	Group1	Group2	n1[#]	n2[#]	statistic	p-value	p-adj.	p.adj.signif
Nasal Wash	AUC_RNA	High-transmissible	Low-transmissible	5	6	-3.0444	0.0023	0.0070	**
Nasal Wash	AUC_RNA	High-transmissible	Non-transmissible	5	15	-3.0045	0.0027	0.0070	**
Nasal Wash	AUC_RNA	Low-transmissible	Non-transmissible	6	15	0.6045	0.5455	0.5455	ns
Air/BC 251	AUC_RNA	High-transmissible	Low-transmissible	5	6	-1.7921	0.0731	0.1462	ns
Air/BC 251	AUC_RNA	High-transmissible	Non-transmissible	5	15	-3.2408	0.0012	0.0036	**
Air/BC 251	AUC_RNA	Low-transmissible	Non-transmissible	6	15	-1.2180	0.2232	0.2232	ns
Air/SPOT	AUC_RNA	High-transmissible	Low-transmissible	5	6	-1.9361	0.0529	0.1586	ns
Air/SPOT	AUC_RNA	High-transmissible	Non-transmissible	5	15	-1.8567	0.0634	0.1586	ns
Air/SPOT	AUC_RNA	Low-transmissible	Non-transmissible	6	15	0.4421	0.6584	0.6584	ns
Nasal Wash	AUC_PFU	High-transmissible	Low-transmissible	5	6	-2.7421	0.0061	0.0122	*
Nasal Wash	AUC_PFU	High-transmissible	Non-transmissible	5	15	-2.9707	0.0030	0.0089	**
Nasal Wash	AUC_PFU	Low-transmissible	Non-transmissible	6	15	0.2616	0.7936	0.7936	ns
Air/BC 251	AUC_PFU	High-transmissible	Low-transmissible	5	6	-1.2986	0.1941	0.1941	ns
Air/BC 251	AUC_PFU	High-transmissible	Non-transmissible	5	12	-3.5086	0.0005	0.0014	**
Air/BC 251	AUC_PFU	Low-transmissible	Non-transmissible	6	12	-2.1625	0.0306	0.0612	ns
Air/SPOT	AUC_PFU	High-transmissible	Low-transmissible	5	5	-2.7803	0.0054	0.0128	*
Air/SPOT	AUC_PFU	High-transmissible	Non-transmissible	5	11	-2.8578	0.0043	0.0128	*
Air/SPOT	AUC_PFU	Low-transmissible	Non-transmissible	5	11	0.4024	0.6874	0.6874	ns

Kruskal-Wallis with Dunn's multiple corrections. P-values adjusted using Holm's method. P.adj.signif thresholds are $p > 0.05$ (ns = not significant), < 0.05 (*), < 0.01 (**). [#] Denotes the number of replicates in each group.

Supplementary Table 4. Notable polymerase and hemagglutinin amino acid differences between A(H5N1) viruses.

Virus[#]	Clade/Genotype	PB2			PA		HA (H5/H3 #)*		
		G362E	E627K	M631L	K142N/E	K497R	E186D E190D	L222Q L226Q	S224G S228G
BC/PHL	2.3.4.4b/D1.1	**E**	**K**	M	K	K	E*	Q	G
WA/239	2.3.4.4b/D1.1	**E**	E	M	K	K	E	Q	G
CO/137	2.3.4.4b/B3.13	G	E	**L**	K	R	E	Q	G
CA/147	2.3.4.4b/B3.13	G	E	**L**	K	R	E	Q	G
MI/90	2.3.4.4b/B3.13	G	E	**L**	K	R	E	Q	G
TX/37	2.3.4.4b/B3.13	**E**	**K**	M	**E**	K	E	Q	G
VN/1203	clade 1	**E**	**K**	M	**E**	K	E	Q	G

[#]A/British Columbia/PHL-2032/2024 (BC/PHL), A/Washington/239/2024 (WA/239), A/Colorado/137/2024 (CO/137), A/California/147/2024 (CA/147), A/Texas/37/2024 (TX/37), A/Michigan/90/2024 (MI/90), A/Vietnam/1203/2004 (VN/1203)
*H5 HA (186, 222, 224) and H3 HA (190, 226, 228) is shown.

Supplementary Table 5. Comparison of nasal wash titers and fever kinetics in ferrets inoculated with diverse influenza virus strains.

Virus	Name in the study	Subtype	NW titer[a]	Temp increase (C°)[b]
A/Colorado/137/2024	CO/137	H5N1	6.8 (d3)	2.3 (d1)
A/California/147/2024	CA/147	H5N1	7.6 (d2)	1.7 (d2)
A/Washington/239/2024	WA/239	H5N1	5.3 (d3)	1.8 (d1)
A/British Columbia/PHL-2032/2024	BC/PHL	H5N1	6.7 (d1)	2.2 (d1)
A/Vietnam/1203/2004	VN/1203	H5N1	5.6 (d5)	2.1 (d1)
A/Michigan/90/2024	MI/90	H5N1	6.3 (d3)	2.3 (d1)
A/Anhui/1/2013	Anhui/1	H7N9	6.7 (d2)	1.3 (d1)
A/Anhui-Lujiang/39/2018	AL/39	H9N2	7.8 (d2)	1.2 (d3)
A/Nebraska/14/2019	NE/14	H1N1pdm09	7.9 (d1)	2.0 (d1)

[a]Ferrets were intranasally inoculated with 6 $\log_{10}$ PFU of virus in 1mL. Average maximum nasal wash (NW) titers are expressed as $\log_{10}$ PFU/mL; limit of detection was 10 PFU/mL; median day is in parenthesis.
[b]Average maximum temperature increase over the baseline (37.5-39.0°C); median day is in parenthesis.

References

1. Pulit-Penaloza, J.A. et.al. Advancing A(H5N1) influenza risk assessment in ferrets through comparative evaluation of airborne virus shedding patterns. GitHub. https://doi.org/10.5281/zenodo.17943807 (2025).