

Seasonal Influenza A Virus in Feces of Hospitalized Adults

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In a cohort of hospitalized adults with seasonal influenza A in Hong Kong, viral RNA was frequently (47%) detected in stool specimens. Viable virus was rarely isolated. Viral RNA positivity had little correlation with gastrointestinal symptoms and outcomes. In vitro studies suggested low potential for seasonal influenza viruses to cause direct intestinal infections.

Although influenza predominantly causes respiratory diseases, gastrointestinal signs such as diarrhea and vomiting are not uncommonly reported, particularly among young, hospitalized children (8%–38%) and immunocompromised persons (1–3). Influenza virus RNA has been detected in feces, but its role is unknown (4–7). We investigated fecal viral RNA shedding in a large cohort of hospitalized adults with seasonal influenza A in Hong Kong Special Administrative Region, People's Republic of China. The potential of seasonal influenza viruses to cause direct intestinal infections was examined.

The Study

We conducted a prospective, observational study among adults hospitalized with laboratory-confirmed seasonal influenza A infections during 2006–2009. Hospital admission, diagnosis, and management procedures have been described (8). Briefly, patients were admitted if severe symptoms, respiratory or cardiovascular complications, or exacerbations of underlying conditions developed. When the patients sought care, nasopharyngeal aspirates (NPAs) were collected for diagnosis by using immunofluorescence assay or reverse transcription PCR. Patients with confirmed influenza A were recruited if they were >18 years of age and sought care within 1 week of illness onset. Patients with pandemic (H1N1) 2009 virus infections were excluded and reported separately (9).

After providing written, informed consent, patients were asked to submit 1 stool specimen for viral

RNA detection during hospitalization, regardless of gastrointestinal symptoms. Clinical information was prospectively recorded (8). For comparison, fecal shedding of respiratory syncytial virus (RSV) and parainfluenza virus (PIV) were studied during a 10-month period by using a similar approach. Ethical approval for the study was obtained from the institutional review boards of The Chinese University of Hong Kong.

All stool specimens were subjected to influenza viral RNA detection by using quantitative real-time reverse transcription PCR targeting the matrix gene (6). If positive, virus subtyping was performed by using H1- and H3-specific conventional PCRs. Freshly collected stool specimens during 1 seasonal peak were simultaneously subjected to viral RNA detection and virus isolation by using MDCK cells. Detailed methods for fecal detection of influenza viruses and RSV and PIV are provided in the online Technical Appendix (wwwnc.cdc.gov/EID/pdfs/11-0205-Techapp.pdf).

Lectin histochemical analysis and double immunofluorescence staining were used to study the distribution of influenza virus receptors on human small and large intestinal tissues. An in vitro virus binding study on intestinal tissues was also performed by using inactivated human virus isolates of subtypes H1N1 (A/HongKong/CUHK-13003/2002) and H3N2 (A/HongKong/CUHK-22910/2004) (online Technical Appendix).

A total of 119 hospitalized adults with seasonal influenza A infections were studied (Table 1). Their median age was 71 years (interquartile range [IQR] 57–79 years), and most (66%) had concurrent conditions; ≈5% were profoundly immunosuppressed. Vomiting and diarrhea were reported by 15 (13%) and 7 (6%) patients, respectively. Influenza A viral RNA was detected in 56 of 119 of stool samples, collected at a median interval of 3 days (IQR 3–5 days) from onset (detection rates by study year and virus subtype are shown in Table 2). Detection rate by day from onset ranged from 31% to 63% and showed a trend to decrease toward the end of the week (Figure 1, panel A). Overall, the mean ± SD fecal viral RNA concentration was 4.4 ± 0.8 log₁₀ copies/g of feces and the median (IQR) was 4.2 (3.8–5.0) log₁₀ copies/g of feces; concentrations tended to decrease with longer time elapsed from onset (Figure 1, panel B).

In most (77%) viral RNA-positive samples, further H1- or H3-specific PCRs identified 7 cases as H1 and 36 cases as H3; unsuccessful amplification was associated with lower viral (matrix gene) concentrations (median [IQR] 3.9 [3.8–4.1] vs. 4.4 [3.8–5.1] log₁₀ copies/g of stool; *p* = 0.04). No discrepancy was found between these and the subtyping results of the virus isolates from NPAs. Fecal

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Table 1. Comparisons of baseline clinical and laboratory variables between influenza patients with positive and negative fecal viral RNA detection test results, Hong Kong, 2006–2009*

Patient characteristics	Fecal viral RNA–positive, n = 56	Fecal viral RNA–negative, n = 63	p value
Mean age, y (SD)	65.3 (18.6)	69.9 (13.4)	0.12
Age group, y, no. (%)			0.35†
18–49	7 (13)	6 (10)	
50–65	18 (32)	14 (22)	
>65	31 (55)	43 (68)	
Female sex, no. (%)	33 (59)	30 (48)	0.27
Interval from illness onset to sample collection ≤5 d, no. (%)	52 (93)	53 (84)	0.14
Concurrent condition, no. (%)			
Any	36 (64)	43 (68)	0.65
Major‡	31 (55)	37 (59)	0.72
Virus isolation, nasopharyngeal aspirates	50 (89)	49 (77)	0.06
Signs and symptoms when care was sought, no. (%)			
Fever	48 (86)	53 (84)	1.00
Cough and sputum	39 (74)	45 (76)	0.83
Sore throat	15 (28)	18 (31)	0.84
Rhinorrhea	21 (40)	21 (36)	0.70
Shortness of breath	18 (34)	27 (46)	0.25
Vomiting or diarrhea	10 (18)	9 (14)	0.63
Vomiting	7 (13)	8 (13)	1.00
Diarrhea	5 (9)	2 (3)	0.25
Laboratory parameters when care was sought			
Total leukocyte count, × 10 ⁹ cells/L, median (IQR)	7.3 (5.9–8.9)	7.9 (6.1–10.3)	0.18
Neutrophil count, × 10 ⁹ cells/L, median (IQR)	5.1 (4.2–7.0)	6.0 (4.1–7.7)	0.22
Lymphocyte count, × 10 ⁹ cells/L, median (IQR)	0.8 (0.6–1.1)	0.9 (0.6–1.2)	0.20
Lymphocyte count <1.0 × 10 ⁹ cells/L, no. (%)	37 (66)	29 (46)	0.03
Monocyte count, × 10 ⁹ cells/L, median (IQR)	0.7 (0.5–0.9)	0.7 (0.5–0.9)	0.51
Platelet count, × 10 ⁹ cells/L, median (IQR)	180 (142–228)	196 (156–248)	0.20
Alanine aminotransferase level, IU/L, median (IQR)	19 (13–32)	22 (15–36)	0.54
Antiviral treatment, no. (%)			
Oseltamivir§	35 (63)	42 (67)	0.70
Zanamivir	6 (11)	5 (8)	0.75
Specimen collected after starting antiviral drugs, no. (%)	39 (70)	42 (67)	0.84
Complication, no. (%)			
Any	39 (70)	44 (70)	0.98
Cardiorespiratory¶	29 (52)	38 (60)	0.36
Clinical outcome, no. (%)			
ICU admission	2 (4)	0 (0)	0.22
Death	0	0	NA
Median duration of hospitalization, d (IQR)	6 (4–12)	5 (4–13)	0.50

*Univariate comparisons of categorical and continuous variables were performed by using Fisher exact test and Mann-Whitney U test or Student t test, whenever appropriate. IQR, interquartile range; ICU, intensive care unit; NA, not applicable.

†Fisher 2×3 exact test.

‡Defined as congestive heart failure; cerebrovascular, neoplastic, chronic liver, and renal diseases; diabetes; ischemic heart disease; or use of immunosuppressant drugs (8). Approximately 5% of patients were profoundly immunocompromised.

§Oseltamivir (standard oral regimen, 75 mg 2×/d for 5 d). Amantadine was coadministered to 7 patients during the 2008–09 influenza season.

¶Defined as pneumonia, acute bronchitis, acute exacerbation of chronic respiratory conditions (e.g., chronic obstructive pulmonary disease, asthma); acute coronary syndrome, decompensated heart failure, arrhythmia, or acute cerebrovascular events (8).

viral RNA detection rate and concentrations were similar between H1 and H3 subtypes (Table 2).

Thirty-eight stool samples from 1 seasonal peak were subjected to virus isolation and viral RNA detection. In 10 cases, cytotoxicity occurred (procedure discontinued); in the remaining 28 cases, 12 were viral RNA positive; only 1 showed virus growth. This sample was from an 82-year-old man with dilated cardiomyopathy hospitalized for seasonal

influenza A (H1N1) pneumonia and heart failure; diarrhea was absent.

Among 25 confirmed RSV or PIV infections (median [IQR] age 71 [55–79] years), viral RNA was detected in 5 fecal samples (collected at median [IQR] 4 [3–6] days after onset); none was culture positive. Fecal viral RNA positivity was lower compared with that of seasonal influenza viruses ($p = 0.01$).

Table 2. Fecal detection of seasonal influenza A viral RNA in stool samples, by year of study and virus subtype, Hong Kong, 2006–2009*

Year of study	No. fecal viral RNA–positive/ no. tested (%)	Fecal viral RNA–positive by virus subtype, no. (%)		Fecal viral RNA concentration by virus subtype, log ₁₀ RNA copies/g stool, median (IQR)	
		H1	H3	H1	H3
2006	11/20 (55)	7/7 (100)	2/4 (50)	5.0 (4.6–6.0)	NA
2007	19/35 (54)	0/0	19/31 (61)	NA	4.2 (4.0–5.1)
2008	11/26 (42)	1/5 (20)	10/21 (48)	NA	3.9 (3.7–4.4)
2009	15/38 (39)	5/10 (50)	10/27 (37)	3.9 (3.8–4.1)	3.9 (3.7–4.7)
All	56/119 (47)	13/22 (59)	41/83 (49)	4.6 (3.9–5.2)	4.2 (3.8–5.0)

*Virus subtyping results were unavailable for 9 cases in 2006, 4 cases in 2007, and 1 case in 2009. IQR, interquartile range; NA, not applicable.

Patients with positive and negative fecal viral RNA detection results were compared (Table 1). Positive fecal viral RNA detection was associated with younger age, shorter interval from illness onset to sample collection, lymphopenia, and positive virus isolation. Multivariate logistic regression showed that lymphopenia (adjusted odds ratio 2.36, 95% confidence interval 1.02–5.47; $p = 0.045$) and positive virus isolation in NPAs (adjusted odds ratio 3.76, 95% confidence interval 1.07–13.20; $p = 0.039$) were significant explanatory variables. No significant association was found between fecal viral RNA detection and clinical outcomes. Fecal viral RNA concentrations were also analyzed, and no association with clinical outcomes was found (data not shown), except for a negative correlation with lymphocyte count (Spearman $\rho = -0.37$, $p = 0.047$).

Lectin histochemical analysis showed that sialic acid α 2,6-Gal (human-like influenza virus receptor) was absent

from epithelial surface of small and large intestines and was found only in lamina propria cells. In contrast, sialic acid α 2,3-Gal (avian-like influenza virus receptor) was found on large intestinal epithelial cells and in lamina propria cells. Virus-binding study showed that neither seasonal influenza A (H1N1) nor A (H3N2) virus bind to small and large intestinal epithelial surface, but they bind to a subset of CD45+ leukocytes (Figure 2).

Conclusions

Direct intestinal infection by seasonal influenza viruses seems an unlikely explanation for the frequent fecal detection of viral RNA in the patients reported here. No clinical correlation was shown for RNA positivity (but was shown with lymphopenia and positive virus isolation in NPA, indicating higher virus load), and culture positivity is rare (4,5,10,11). Human-like influenza virus receptor is

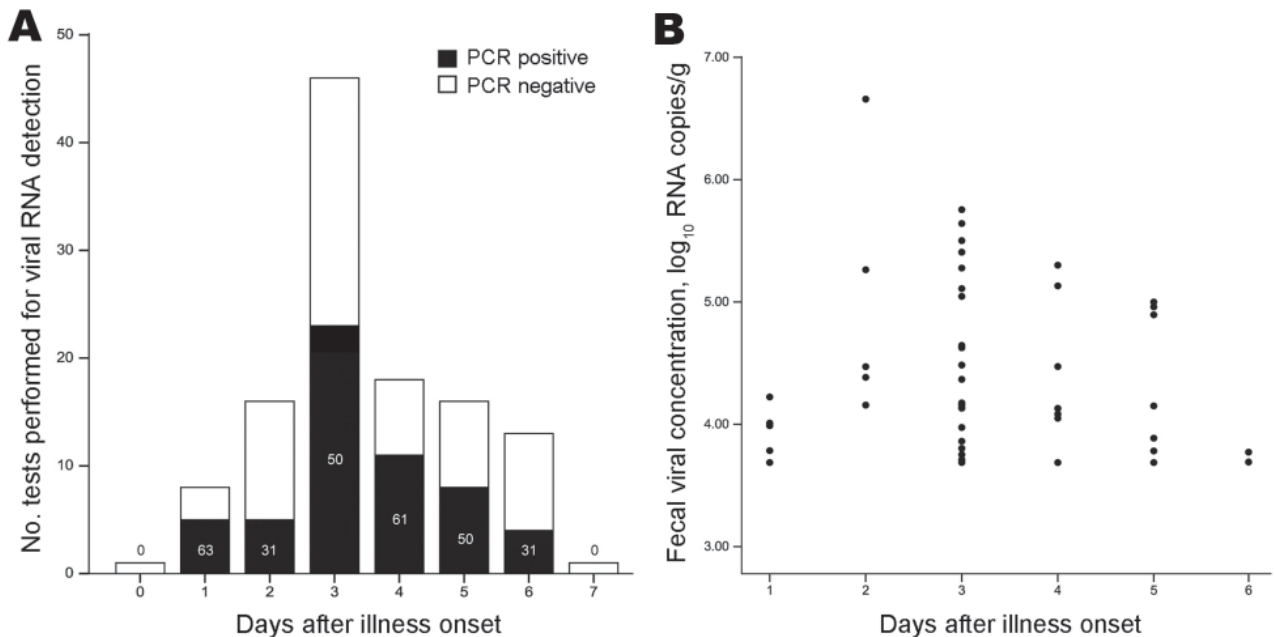


Figure 1. Fecal seasonal influenza A viral RNA detection rate and its concentration, by number of days after illness onset, Hong Kong, 2006–2009. A) Fecal viral RNA detection rate. Numbers in bars represent percentage of cases with positive viral RNA detection. B) Fecal viral RNA concentration. Three outliers were omitted from the figure for better illustration. Fecal viral RNA concentration was determined by using quantitative real-time reverse transcription PCR specific for the viral matrix gene and was expressed as log₁₀ RNA copies/g of stool. The lower detection limit of the assay was 3.7 log₁₀ RNA copies/g of stool.

not found to express on normal intestinal epithelial cells (12). These findings agree with reports which showed that intestinal cells and tissues do not support efficient replication of seasonal viruses (12,13), thus their low potential to cause direct intestinal infection. Alternatively, swallowing of virus-containing nasopharyngeal secretions (although it seems inadequate to explain the higher rate of detecting

fecal viral RNA than RSV or PIV) and hematogenous dissemination to organs through infected lymphocytes or macrophages in severe influenza cases with high virus load (spillover) are possible explanations for fecal viral RNA detection (2,14). Our findings on virus receptor distribution and in vitro virus binding to intestinal lamina propria leukocytes lends support to the latter hypothesis. Notably, viral RNA positivity in nonpulmonary tissues infiltrating mononuclear cells without detectable viral particles or antigens or tissue damage has been reported (15). Our study does not reject the possibility of seasonal influenza viruses causing occasional, disseminated infection in profoundly immunosuppressed persons because receptor affinity is not absolute (2). Conversely, highly pathogenic avian influenza (H5N1) and pandemic (H1N1) 2009 viruses have the ability to bind to avian-like influenza virus receptors on colonic epithelium and to replicate efficiently in intestinal cells and tissues (12). Their enhanced potential to cause direct intestinal infections and fecal–oral transmission deserve further investigation.

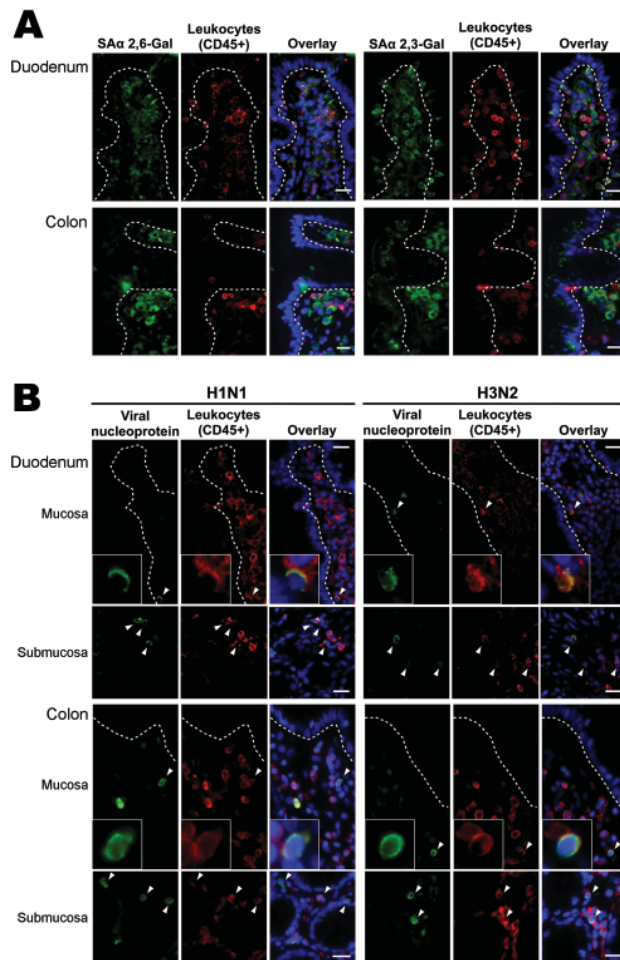


Figure 2. Intestinal distribution of influenza virus receptors and in vitro binding of inactivated seasonal influenza A (H1N1) and A (H3N2) viruses to human duodenal and colonic tissues. Images in the panels labeled Overlay show the green, red, and blue (nuclei counterstain) color channels in the same view. Dotted lines outline basal lining of intestinal epithelium. Arrowheads denote virus-bound cells. Scale bars = 20 μ m. A) Double immunofluorescence staining showing that human-like influenza virus receptor sialic acid (SA) α 2,6-Gal; green) was not found on epithelial surface of small and large intestines but in lamina propria cells. Avian-like influenza virus receptor (SA α 2,3-Gal; green) was found on colonic epithelial surface and in lamina propria cells. Part of receptor-positive cells coexpressed CD45 (leukocyte common antigen; red), representing leukocytes. B) In vitro virus binding showing that neither seasonal influenza A (H1N1) nor A (H3N2) viruses bind to epithelial surface of small and large intestines but only to a subset of intestinal CD45+ leukocytes interspersed in the lamina propria and submucosa.

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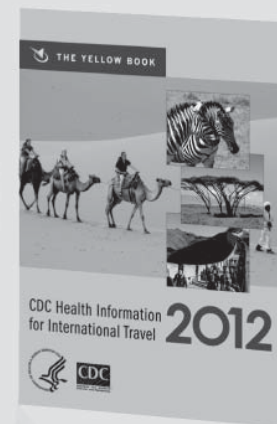
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Technical Appendix

Fecal Detection and Isolation of Influenza A Virus

Detection and quantification of fecal influenza A viral RNA were performed by quantitative real-time reverse transcription PCR (RT-PCR) specific for the viral matrix-gene as described (1–3). Briefly, viral RNA was extracted (in batches of 5–20 frozen [–80°C] specimens) from a 10% stool suspension in phosphate-buffered saline (100 mg of stool plus 900 µL of saline) by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA). Synthesis of complementary DNA from purified RNA was performed by using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and random hexamers. Fecal viral RNA concentration was determined against 10-fold serially diluted plasmid standards prepared from cloning the amplicon into pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA). The lower limit of detection of the assay is 3.7 log₁₀ RNA copies per gram of stool (log₁₀ copies/g stool). In addition, virus subtyping was performed on the viral RNA–positive specimens by using H1- and H3-specific conventional PCRs as described (4).

To minimize specimen contamination, aerosol-resistant tips were used in all liquid pipetting throughout the experiments; negative controls without stool in viral RNA extraction and negative controls without template in RT-PCR steps were included in every run; and PCR master mixture preparation, template addition, and PCR were performed in 3 separate, designated areas. During PCR setup, reaction tubes containing specimen materials were capped before adding plasmid standards to control tubes. No false-positive results were observed in any negative controls.

Fresh stool specimens collected from influenza patients during 1 seasonal peak were simultaneously subjected to virus isolation. Briefly, a 10% stool suspension in viral transport medium was prepared immediately after specimen collection. After centrifugation, the suspension was sterilized through a 0.45-µm filter and adjusted to physiologic pH range. MDCK

cells were then inoculated with the stool filtrate at 37°C for 1 hour, rinsed, and incubated at 33°C on a roller drum for 10–14 days. Cells were monitored daily for evidence of cytotoxicity and cytopathic effect. Detection of viral antigen was performed by using an immunofluorescence assay.

Fecal Detection and Isolation of Respiratory Syncytial Virus and Parainfluenza Virus

An approach similar to that of seasonal influenza virus detection was used for fecal virus detection among respiratory syncytial virus (RSV)– or parainfluenza virus (PIV)–infected patients. Briefly, stool specimens were processed as described above. Detection of fecal viral RNA was performed by using TaqMan-based RT-PCRs specific for the nucleocapsid gene of RSV and hemagglutinin-neuraminidase genes of PIV (5). Virus isolation was performed by using HEp-2 cells for RSV and LLC cells for PIV. Viral antigen was detected by virus-specific immunofluorescence assay.

Double Immunofluorescence Staining for Influenza Virus Receptors and Surface Marker of Receptor-positive Cells in Human Small and Large Intestinal Tissues

Archived formalin-fixed, paraffin-embedded human adult normal duodenal and colonic biopsy tissues obtained from 6 persons (3 duodenal and 3 colonic, all without influenza infection) were used for virus receptors distribution study. Sections of 5- μ m thickness were prepared from tissue blocks, deparaffinized in xylene, and rehydrated in sequential ethanol gradient (from 100% to 70%). Antigen retrieval was performed by boiling the sections in 10 mmol/L citrate buffer for 15 minutes. After cooling, sections were incubated with Image-iT FX signal enhancer (Invitrogen) for 30 minutes, followed by protein block (Dako, Roskilde, Denmark) for 30 minutes and avidin/biotin blocking kit reagents (Vector Laboratories, Burlingame, CA, USA) as per manufacturer's instruction.

Sections were double-labeled by incubating with 10 μ g/mL of biotinylated *Sambucus nigra* lectin (against human influenza virus receptor sialic acid α -2,6 galactose) or *Maackia amurensis* lectin I (against avian influenza virus receptor sialic acid α -2,3 galactose; Vector Laboratories), and mouse anti-human monoclonal antibody against CD45 (leukocyte common antigen) (Dako) at 4°C overnight (14–18 hours). Sections were then incubated with 2 μ g/mL of Alexa Fluor 488–conjugated streptavidin (Invitrogen) and Alexa Fluor 594–conjugated goat anti-mouse immunoglobulin (Invitrogen) for 60 minutes, and mounted with ProLong Gold antifade

reagent containing 4',6-diamidino-2-phenylindole (Invitrogen). Images of each color channel were captured by using epifluorescence microscope. To test the specificity of the staining, negative controls using protein block reagent in replacement of the lectin, and isotype-matched control in replacement of the primary antibody were included. Neighboring sections were used for the controls. Sections were washed with phosphate-buffered saline with or without Tween20 between steps, where appropriate. All steps were performed at room temperature unless otherwise specified.

In Vitro Binding of Influenza A Viruses to Human Small and Large Intestinal Tissues

Human isolates of seasonal influenza A H1N1(A/HongKong/CUHK-13003/2002) and H3N2(A/HongKong/CUHK-22910/2004) viruses were propagated in MDCK cells, and virus-containing supernatants were inactivated in 0.2% formalin for at least 3 days. Inactivated viruses were concentrated by using Amicon Ultra-4 (100 kDa) centrifugal filter unit (Millipore) and then dialyzed against phosphate-buffered saline. In vitro virus binding was performed on formalin-fixed, paraffin-embedded tissues. Tissue sections were processed as described above and then incubated with the viruses (3×10^4 RNA copies) at 4°C overnight. Monoclonal antibodies against viral nucleoprotein (clone A1 for subtype H1N1 and clone A3 for subtype H3N2, 10 µg/mL; Millipore, Billerica, MA, USA) were used to detect the viruses. Double immunofluorescence staining for the viruses and CD45 was performed by using the Vector M.O.M. system as per manufacturer's instructions (Vector Laboratories). Signals were visualized by using Alexa Fluor 488 for viral nucleoprotein and Alexa Fluor 594 for CD45.

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