Highly Pathogenic Avian Influenza A(H5N8) Clade 2.3.4.4b Virus in Dust Samples from Poultry Farms, France, 2021

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Avian influenza A(H5N8) virus has caused major epizootics in Europe since 2016. We conducted virologic analysis of aerosol and dust collected on poultry farms in France during 2020–2021. Our results suggest dust contributes to viral dispersal, even early in an outbreak, and could be a valuable surveillance tool.

Avian influenza is a viral disease caused by influenza A viruses, segmented, negative, single-stranded RNA viruses belonging to the *Orthomyxoviridae* family. Wild aquatic birds are the virus reservoir and generate occasional worldwide panzootic outbreaks during seasonal migrations (*1*). Highly pathogenic avian influenza (HPAI) virus subtypes can cause panzootic outbreaks associated with high mortality in wild and domestic birds, as well as substantial economic losses for the poultry industry, and are a major threat to public health because of their zoonotic potential.

During winter 2020–21, the HPAI H5N8 virus belonging to the A/goose/Guangdong/1/1996 clade 2.3.4.4b lineage caused hundreds of outbreaks among wild and domestic flocks across Europe (*2*,*3*). France was severely affected; 492 poultry farms, primarily duck farms, were infected during December 5, 2020–May 3, 2021. Despite reinforced surveillance activities, the virus spread rapidly, posing major challenges for surveillance and control. Officially recognized surveillance methods involve tracheal or cloacal swab-based sampling (*4*,*5*). However, these methods are laborious and have technical requirements that make application on such a massive scale difficult; thus, newer surveillance methods are needed.

Epidemiologic modeling of this outbreak suggested within-farm viral transmission was extremely fast, and the environment was a major source of contamination for neighboring farms (*6*). HPAI viruses disperse in aerosols, in fomites carried by human and animal vectors, and via feathers, fecal particles, and to a great extent, dust (*7*–*9*). Poultry farms are known to heavily generate dust particles that spread from feed, litter, feces, and animal skin and feathers (*9*,*10*). These particles can act as vehicles for bacteria and viruses and are classified, depending on their size, as inhalable (<100 μm), thoracic (<10 μm), or respirable (<4 μm) (*10*). In poultry houses, most dust consists of nonrespirable particles >4 μm (*10*). We evaluated the role of dust as a vehicle of H5N8 clade 2.3.4.4b virus and assessed whether dust or aerosol sampling is a viable alternative to bird swab sampling for HPAI virus surveillance.

The Study

During December 2020–April 2021, we conducted a study in 63 poultry houses located in 4 departments (administrative units) in France highly affected by HPAI H5N8 virus outbreaks. On the basis of daily official outbreak reports, we identified HPAI-infected poultry houses and poultry houses in close vicinity or with epidemiologic links to infected houses. The study included a total of 48 duck houses, 12 chicken houses, 2 quail houses, and 1 goose house. We selected farms identified as being near an HPAI outbreak to reflect a range of sanitary statuses and infection stages (i.e., no, mild, or severe clinical signs; high mortality rates). We specifically included houses without

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clinical signs among animals to evaluate virus dispersal and dust testing for HPAI surveillance in the early stages of infection.

In each selected poultry house, we collected surface dust with 2 wipes on the building's walls and feeders (*9*,*11*) (Appendix, https://wwwnc.cdc.gov/EID/article/28/7/21-2247-App1.pdf). In 19 houses, we also collected aerosol samples by using 2 devices, Coriolis Compact (Bertin Instruments, https://www.bertin-instruments.com) and the NIOSH BC 251 developed by the National Institute for Occupational Safety and Health (NIOSH; https://www.cdc.gov/niosh) (Appendix). Furthermore, we collected tracheal swab samples from 20 randomly selected birds in each house (Appendix Table 1). We chose tracheal over cloacal swab samples because the typical respiratory shedding and tropism of HPAI H5N8 clade 2.3.4.4 viruses enables earlier detection in the respiratory tract than cloacae (*12*,*13*).

We performed real-time quantitative reverse transcription PCR on all samples to detect HPAI virus at the molecular level by targeting the matrix protein and H5 genes (Appendix). We compared cycle threshold (Ct) distributions of each sample by using raincloud plots and a boxplot model (Figure 1). In general, Ct values for tracheal swabs (≈25.2) and dust (≈28.6) were similar (Figure 1; Appendix). Between the 2 aerosol collectors, the Coriolis device showed more positive results (Ct <40) than the NIOSH BC 251 sampler. Furthermore, we noted HPAI H5N8

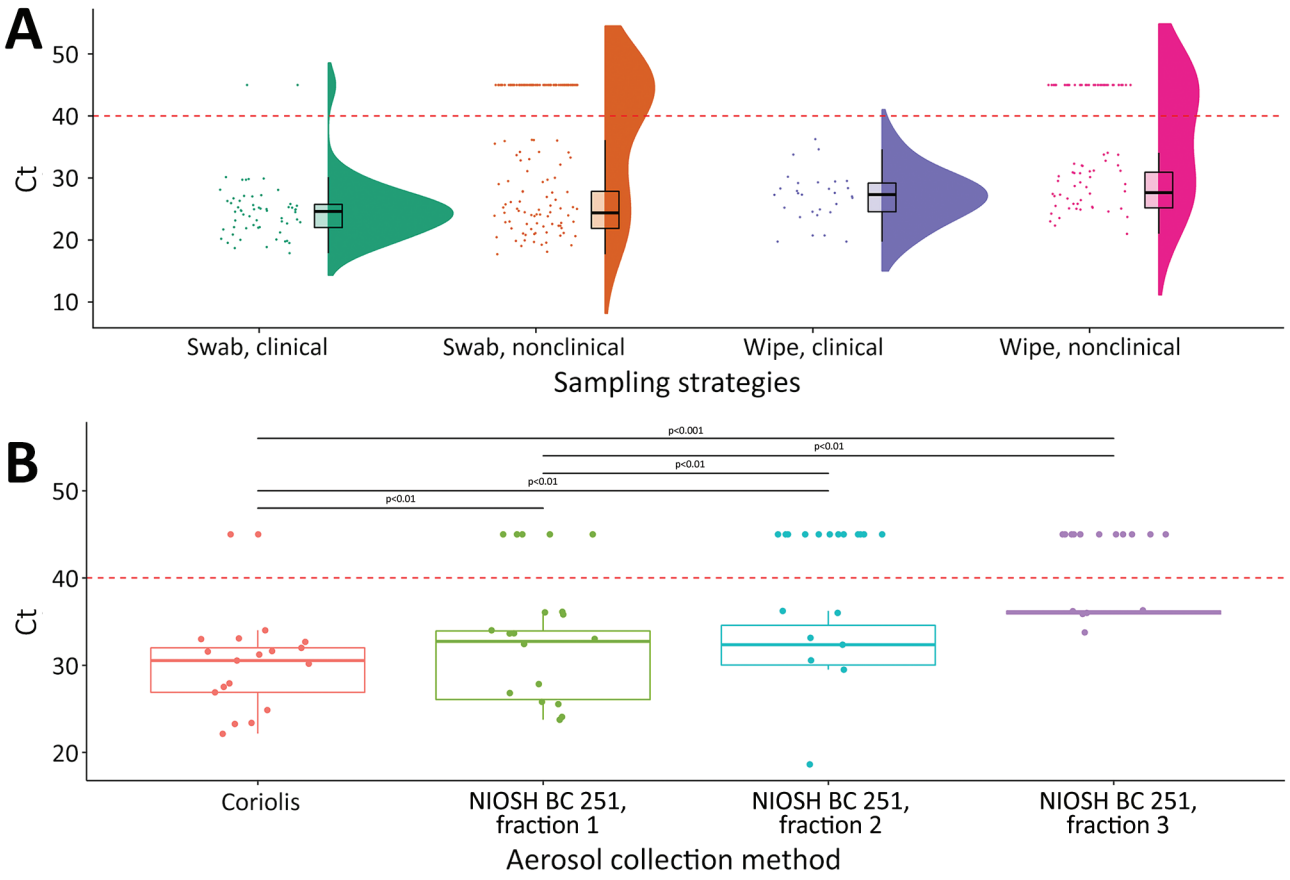

Figure 1. Ct values of highly pathogenic avian influenza A(H5N8) clade 2.3.4.4b virus detected by real-time quantitative reverse transcription PCR from tracheal swab and environmental samples collected on poultry farms, France, December 2020–April 2021. We used a Wilcoxon test for statistical analysis and considered samples with Ct ≤40 negative. Each dot indicates a Ct value from 1 wipe sample or 1 pool of 5 tracheal swab samples. Box plots show 95% CI for Ct values; horizontal lines in boxes indicate means and error bars SDs. Red dashed horizontal lines indicate Ct of 40, the cutoff value for negative results. A) Half-violin, scatter, and box plots of Ct values for samples collected by using tracheal swab samples or surface wipe samples from 63 poultry houses with and without clinical signs among animals. Half-violins show distribution of Ct values for each sample type. B) C_t values for aerosol samples collected in 19 poultry houses. Aerosol samples were collected by using the Coriolis Compact (Bertin Instruments, https://www.bertin-instruments.com) and the NIOSH BC 251 (https://www.cdc.gov/niosh). The NIOSH BC 251 sampling device has 3 fractions for different particle sizes; fraction 1 for >4 μm, fraction 2 for 1–4 μm, and fraction 3 for <1 μm. Ct, cycle threshold; NIOSH, National Institute for Occupational Safety and Health.

Table 1. Estimated sensitivity of sampling methods and sampling strategies by latent class analysis for detection of highly pathogenic avian influenza A(H5N8) virus on poultry farms, France, December 2020–April 2021

Clinical signs	Samples*	Estimated sensitivity of sampling method (95% credible interval)†	Estimated sensitivity of sampling strategy (95% credible interval)‡
Clinical signs in flock	Tracheal swab	0.77 (0.44–0.99)	1.00 (0.90–1.00)
	Wipe	0.89 (0.64–1.00)	0.99 (0.87–1.00)
	Coriolis	0.93 (0.69–1.00)	0.93 (0.69–1.00)
	NIOSH BC 251	0.93 (0.69–1.00)	0.93 (0.69–1.00)
No clinical signs in flock	Tracheal swab	0.46 (0.15–0.97)	0.92 (0.48–1.00)
	Wipe	0.90 (0.67–1.00)	0.99 (0.89–1.00)
	Coriolis	0.92 (0.63–1.00)	0.92 (0.63–1.00)
	NIOSH BC 251	0.67 (0.34–0.91)	0.67(0.34–0.91)

*Each farm or building was sampled by using 20 tracheal swab samples (pooled in sets of 5 for RT-PCR) and 2 wipe samples from surfaces; on 19 farms we also collected 1 air sample from each of the 2 aerosol collection devices, the Coriolis Compact (Bertin Instruments, https://www.bertin-instruments.com) and the NIOSH BC 251 developed by the National Institute for Occupational Safety and Health (https://www.cdc.gov/niosh).
†Sampling method relates to the simple analysis of individual samples; individual tracheal swab samples are those analyzed in pools of 5 samples; thus, the sensitivity of the sampling method corresponds to the probability that a single sample, or a pooled sample for the tracheal swabs, tests positive in an infected poultry house.
‡Sampling strategy relates to the combined analysis of the different individual samples at the farm or building level, assuming that the farm or building is positive when ≥1 individual sample tests positive; thus, the sensitivity of the sampling strategy corresponds to the probability that ≥1 sample, or ≥1 pool of 5 tracheal swab samples, tests positive in an infected poultry house.

virus was more easily detected in the largest particles, those ≥1 μm (Figure 1). These results suggest that the HPAI H5N8 virus dispersion is associated with large dust particles, which could be a major vehicle for viral spread.

To estimate the sensitivity of the 4 different sampling methods (tracheal swab samples, surface wipes, and Coriolis and NIOSH aerosol samplers) in houses with or without poultry showing clinical signs, we used a latent class modeling approach, necessary when no standard has been established (*14*). We adjusted the model to cross-detect each farm by the 4 different sampling methods and estimated model parameters in a Bayesian framework (Appendix). Model outputs suggested that the different sampling methods had equivalent sensitivity in HPAI-infected flocks showing clinical signs. Surface dust and aerosol sampling showed substantially higher sensitivity in HPAI-infected flocks without clinical signs, but the difference was not statistically significant despite overlap of 95% credible intervals (Table 1; Figure 2).

Finally, to assess the infectiousness of environmental samples, we processed 25 surface dust or aerosol samples taken from 5 animal houses and used these for virus isolation in embryonated eggs (Appendix). Among 25 samples, 12 (48%) tested positive,

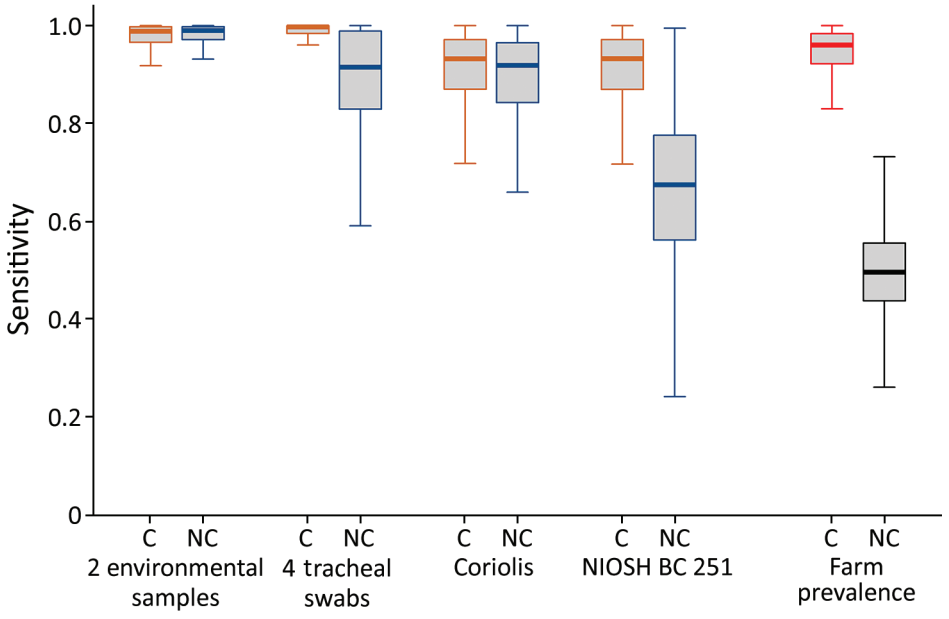

Figure 2. Sensitivity comparison of 4 sampling techniques used to detect highly pathogenic avian influenza A(H5N8) clade 2.3.4.4b virus from 63 poultry farms, France, December 2020–April 2021. Sampling was conducted in poultry houses with and without clinical signs among flocks. Box plots show 95% CIs; horizontal lines in boxes indicate means, error bars SDs. The 2 environmental samples refer to 2 wipes collected in the animal houses, 1 on feeders and 1 on walls. Tracheal swab samples refer to 4 pools of 5 swab samples collected per house. Aerosol samples were collected from 19 poultry houses by using the Coriolis Compact (Bertin Instruments, https://www.bertin-instruments.com) and the NIOSH BC 251 (https://www.cdc.gov/niosh). The NIOSH BC 251 sampling device has 3 fractions for different particle sizes; fraction 1 for >4 μm, fraction 2 for 1–4 μm, and fraction 3 for <1 μm. Farm-level disease prevalence was 0.96 for houses in which animals had clinical signs and 0.5 in houses in which animals did not have clinical signs. C, clinical signs; NC, no clinical signs; NIOSH, National Institute for Occupational Safety and Health.

Table 2. Viral isolation assays on chicken embryonated eggs performed on 5 of the 63 poultry houses in a study to detect highly pathogenic avian influenza A(H5N8) virus on poultry farms, France, December 2020–April 2021*

	House 11		House 26		House 29		House 30		House 34	
Sample type†	Ct	VI	Ct	VI	Ct	VI	Ct	VI	Ct	VI
Tracheal swab	25	+	20.7	+	21.9	+	18.9	+	20	+
Dust wipe, feeders	25.8	–	25.1	–	27.4	+	29.5	+	24.2	+
Dust wipe, walls	27.5	+	25.5	–	30.1	+	28.3	+	23	+
Coriolis	32	–	33.6	–	27.8	–	25.8	+	26.9	+
NIOSH BC251										
Fraction 1	34	–	33.6	–	27.8	–	25.8	+	23.7	+
Fraction 2	–	ND	36	–	32.4	–	33.1	–	18.6	+
Fraction 3	–	ND	–	ND	36.3	–	–	ND	–	ND

*Ct, cycle threshold; ND, not done; VI, virus isolation; +, positive; –, negative.
†Each farm or building was sampled by using 4 pools of 5 tracheal swab samples, 2 wipe samples (1 from feeders, 1 from walls), and on 19 farms, 1 air sample from each of the 2 aerosol collection devices, the Coriolis Compact (Bertin Instruments, https://www.bertin-instruments.com) and the NIOSH BC 251, developed by the National Institute for Occupational Safety and Health (https://www.cdc.gov/niosh). NIOSH BC 251 sampling device has 3 fractions for different particle sizes; fraction 1 for >4 µm, fraction 2 for 1–4 µm, and fraction 3 for <1 µm.

confirming that viral isolation is possible from these sampling methods (Table 2).

Conclusions

We used field conditions to evaluate whether dust from poultry farms contained HPAI viruses and to compare surface dust and aerosol testing for HPAI virus against official swab-based methods. We used wipe tests to collect surface dust and 2 bioaerosol devices to collect aerosol samples during the 2020–21 HPAI H5N8 virus epizootic outbreak in France. Standard molecular analysis detected high viral RNA loads in the early phase of flock infection, before clinical signs appeared. In addition, size fractioning of aerosol samples revealed that high RNA viral loads and infectious viral particles were associated with the largest particles (>1 µm), which are easy to collect and use for molecular analysis. However, the field conditions we used cannot be reproduced in experimental animal trials because of ethical and biosecurity requirements, which result in dramatically lower dust loads than those found in the field.

Recent research on influenza transmission routes revealed that nonrespiratory airborne particles are more likely to cause infection than are droplets or fomites (*7*). Infectious aerosols generated from inert objects handled by humans or dispersed through animal movements can lead to further infection. Dust can carry infectious particles and is omnipresent in poultry houses (*10*) and so could be a major means of viral transmission and dispersal in the environment. These findings suggest that biosecurity protocols should strongly emphasize limiting the amount of dust dispersed via farm equipment to reduce the spread of HPAI viruses.

Of note, for early detection, before flock animals show clinical signs of illness, we found that surface dust sampling using wipe tests and aerosol sampling using a high flow rate collection device are more sensitive than tracheal swab samples. The higher sensitivity of environmental sampling methods for early detection is likely because of infection dynamics at the flock level. During the early phases of infection, only a few animals are infectious, making the probability of detecting virus during individual swab-based sampling low (*6*). Swab sampling also is time consuming, labor-intensive, and expensive, whereas dust wiping is inexpensive, fast, easy to perform, and noninvasive.

In conclusion, we detected HPAI H5N8 clade 2.3.4.4b virus in dust samples from poultry farms during a large epizootic in France. Our findings suggest dust wipe samples are an efficient surveillance tool and could enable more rapid virus detection and implementation of measures to curb virus spread.

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Mr. Filaire is an engineer in biosciences and a PhD candidate in a collaborative project between the Host-Pathogen Interactions Joint Research Unit, National Veterinary School and INRAe, Toulouse, France; EIP Purpan, Toulouse; and Theseo, Lanxess Group, Laval, France. His research focus is on innovative methods for detection and characterization of emerging viruses.

References

1. Munster VJ, Baas C, Lexmond P, Waldenström J, Wallensten A, Fransson T, et al. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS Pathog. 2007;3:e61. https://doi.org/10.1371/journal.ppat.0030061
2. Lewis NS, Banyard AC, Whittard E, Karibayev T, Al Kafagi T, Chvala I, et al. Emergence and spread of novel H5N8, H5N5 and H5N1 clade 2.3.4.4 highly pathogenic avian influenza in 2020. Emerg Microbes Infect. 2021;10:148–51. https://doi.org/10.1080/22221751.2021.1872355
3. European Food Safety Authority; European Centre for Disease Prevention and Control and European Union Reference Laboratory for Avian Influenza; Adlhoch C, Fusaro A, Kuiken T, Niqueux É, Staubach C, Terregino C, et al. Avian influenza overview May–August 2020. EFSA J. 2020;18:e06270. PubMed https://doi.org/10.2903/j.efsa.2020.6270
4. World Organisation for Animal Health (OIE). Avian influenza (including infection with high pathogenicity avian influenza viruses). In OIE terrestrial manual 2021. Geneva: The Organisation; 2021.
5. Nielsen SS, Alvarez J, Bicout DJ, Calistri P, Depner K, Drewe JA, et al.; EFSA Panel on Animal Health and Welfare (EFSA AHAW Panel). Scientific opinion on the assessment of the control measures of the category A diseases of Animal Health Law: Highly Pathogenic Avian Influenza. EFSA J. 2021;19:e06372. https://doi.org/10.2903/j.efsa.2021.6372
6. Vergne T, Gubbins S, Guinat C, Bauzile B, Delpont M, Chakraborty D, et al. Inferring within-flock transmission dynamics of highly pathogenic avian influenza H5N8 virus in France, 2020. Transbound Emerg Dis. 2021;68:3151–5. https://doi.org/10.1111/tbed.14202
7. Asadi S, Gaaloul ben Hnia N, Barre RS, Wexler AS, Ristenpart WD, Bouvier NM, et al. Influenza A virus is transmissible via aerosolized fomites. Nat Commun. 2020; 11:4062. https://doi.org/10.1038/s41467-020-17888-w
8. Spekreijse D, Bouma A, Koch G, Stegeman A. Quantification of dust-borne transmission of highly pathogenic avian influenza virus between chickens. Influenza Other Respir Viruses. 2013;7:132–8. https://doi.org/10.1111/j.1750-2659.2012.00362.x
9. Lopez KM, Nezworski J, Rendahl A, Culhane M, Flores-Figueroa C, Muñoz-Aguayo J, et al. Environmental sampling survey of H5N2 highly pathogenic avian influenza–infected layer chicken farms in Minnesota and Iowa. Avian Dis. 2018;62:373–80. https://doi.org/10.1637/11891-050418-Reg.1
10. Zhao Y, Aarnink AJA, De Jong MCM, Groot Koerkamp PWG. Airborne microorganisms from livestock production systems and their relation to dust. Crit Rev Environ Sci Technol. 2014; 44:1071–128. https://doi.org/10.1080/10643389.2012.746064
11. Carrique-Mas JJ, Breslin M, Sayers AR, McLaren I, Arnold M, Davies R. Comparison of environmental sampling methods for detecting *Salmonella* in commercial laying flocks in the UK. Lett Appl Microbiol. 2008;47:514–9. https://doi.org/10.1111/j.1472-765X.2008.02450.x
12. Gaide N, Foret-Lucas C, Figueroa T, Vergne T, Lucas MN, Robertet L, et al. Viral tropism and detection of clade 2.3.4.4b H5N8 highly pathogenic avian influenza viruses in feathers of ducks and geese. Sci Rep. 2021;11:5928. https://doi.org/10.1038/s41598-021-85109-5
13. Beerens N, Germeraad EA, Venema S, Verheij E, Pritz-Verschuren SBE, Gonzales JL. Comparative pathogenicity and environmental transmission of recent highly pathogenic avian influenza H5 viruses. Emerg Microbes Infect. 2021;10:97–108. https://doi.org/10.1080/22221751.2020.1868274
14. van Smeden M, Naaktgeboren CA, Reitsma JB, Moons KG, de Groot JA. Latent class models in diagnostic studies when there is no reference standard—a systematic review. Am J Epidemiol. 2014;179:423–31. https://doi.org/10.1093/aje/kwt286
15. Raynor PC, Adesina A, Aboubakr HA, Yang M, Torremorell M, Goyal SM. Comparison of samplers collecting airborne influenza viruses: 1. primarily impingers and cyclones. PLoS One. 2021;16:e0244977. https://doi.org/10.1371/journal.pone.0244977

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Appendix

Methods

Sampling on Farms

Tracheal swab samples were taken and tested for avian influenza virus (AIV) by using official M/H5 PCR kits and procedures. H5 PCR-positive samples were confirmed as highly pathogenic avian influenza (HPAI) A(H5N8) clade 2.3.4.4.b virus by the French National Laboratory for Avian Influenza and Newcastle Disease using the official procedures. On-farm investigations and collection and shipping of samples were performed in strict compliance with regulation and biosecurity procedures, with the authorization and supervision of official veterinary services.

Dust was collected using dry wipes, of ≈900 cm^2 (Grosseron, https://www.grosseron.com), on the building's walls and, in 51/63 farms, on feeders. The aim was to collect dust without feces, litter, or food residues to avoid PCR inhibition as much as possible. Therefore, for feeders, the food distributing pipes were preferred for automatic feeders, otherwise the dust was collected in the top part of feeders to avoid feces or food residues. On walls, the dust was sampled on all surfaces roughly above 60 cm high, which appears not to have any other particles except dust. Both sides of the wipes must be covered by dust. Wipes were shipped to the National Veterinary School of Toulouse (France), stored at 4°C, and processed within 48 hours.

Aerosol sampling using the dry cyclonic air sampler Coriolis Compact (Bertin Technologies, https://www.bertin-instruments.com) was done according to the manufacturer's instructions. In brief, the Coriolis Compact was calibrated at a 50 L/min air flow rate that enables

the dry collection of aerosol particles from 500 nm–10 µm in diameter. The 2-stage bioaerosol cyclone (BC) sampler, NIOSH BC 251, developed by the National Institute of Occupational Safety and Health (NIOSH; https://www.cdc.gov/niosh), was also used according to the manufacturer's instructions. Prior to utilization, the sampler, connected to an APEX (Casella, https://www.casella.com) personal sampling pump, was calibrated using a flow meter at 3.5 L/min. On the sampler, 15 mL and 1.5 mL collection tubes were installed, as well as the cassette in which a handmade 37 mm diameter polytetrafluoroethylene (PTFE) filter with 1.5 µm pore was installed.

During the collection time, both samplers were positioned roughly in the center of the barn at ≈1.5 m from the floor and at a minimal distance of 2 m from each other to avoid interference. The simultaneous use of both collection devices with respectively high (50 L/min) and low (3.5 L/min) air flow rates was shown to enable an accurate airborne virus detection and quantification (*1*). Aerosols were collected for 20 min with the Coriolis sampler, allowing aerosol collection of 1 m^3 of air, and between 25 and 60 min for the NIOSH samplers due to experimental constraints. All experimental samples were stored at 4°C before being processed.

Processing Methods

Prior to RNA extraction, tracheal swab and environmental samples (dust and aerosols) were processed. Tracheal swab samples were individually placed into single 1.5 mL centrifuge tubes containing 500 µL phosphate-buffered saline (PBS) and vigorously vortexed for 10–15 s. From the 20 swabs, we created 4 pools of 5 swabs by using 100 µL of each sample. Dust samples from the wipes were processed by using 20 mL PBS directly in the transport bag. After mixing by hand massage for 2–3 min, the dust solution was collected and aliquoted into 1.5 mL centrifuge tubes.

Aerosols were resuspended by using a PBS-0.5% BSA (w/v) solution; 1 mL of the solution was added into the Coriolis Compact collection cone and the first 2 stages of the NIOSH BC 251 collection tubes (15 mL falcon tubes and 1.5 mL microtubes). All samples were vigorously vortexed for 10–15 s before being aliquoted into 1.5 mL centrifuge tubes. The NIOSH BC 251 fraction 3 membrane filter was carefully collected from the cassette by using sterile pliers and placed into a 50 mL falcon. The filter was vortexed for 10 s while dry before adding 1.5 mL of the PBS-0.5% BSA solution and submitted through another vigorous vortex for

10–15 s. Samples were aliquoted into 1.5 mL centrifuge tubes. All samples were stored at −80°C after processing.

RNA Extraction and PCR Methods

RNA samples were extracted by using the magnetic bead-based ID Gene Mag Fast Extraction Kit (IDvet, https://www.id-vet.com) associated with the IDEAL 32 extraction robot (IDvet), following the manufacturer's instructions. The presence of AIV RNA was investigated by performing a 1-step, real-time reverse transcription quantitative PCR (rRT-qPCR), Influenza A Duplex kit (IDvet), targeting the matrix (M) gene and then targeting the H5 subtype from positive results by using the Influenza H5/H7 Triplex kit (IDvet).

Virus Isolation

Positive H5 subtype rRT-qPCR biologic and environmental samples from 5 different animal houses (A, B, C, D, E) were selected, based on their cycle threshold (C_t) values and global study representativity, to test for virus viability using specific virus free (SPF) embryonated chicken eggs purchased from INRAE PFIE (https://www.nadir-project.eu). SPF eggs were incubated for 9–11 days at 37°C. Infection was executed in triplicate to optimize data analysis. Different inoculum concentrations were used depending on the sampling method. Inoculum from dust sampling (walls and feeders) were diluted at 0.1 and aerosols from the NIOSH BC 251 sampler were diluted at 0.5. Inoculum from the Coriolis Compact was used at a concentration of 0.5 and a single pool of tracheal swab samples was used at a concentration of 0.01 for a single egg, and a concentration of 0.1 for the last 2 remaining eggs. All dilutions were achieved by using a sterile 1× PBS solution with penicillin (1,000 U/mL) and streptomycin (1 mg/mL). Eggs were inoculated with 150 µL of the correspondent dilution, kept in a humidity-chamber at 37°C for 48 h then at 4°C for 12 h. Allantoic fluid was collected from each egg and a hemagglutination titration was directly performed in a 96-well U-bottom plate; 100 µL of allantoic fluid was pipetted in the first plate row then the next 7 rows were filled with 50 µL PBS. A cascade of 0.5 dilutions was performed and 50 µL of 1% solution of fresh and washed chicken red blood cells were added to each well before a 25 min room temperature incubation. All samples were controlled by H5 AIV subtype rRT-qPCR by using the ID Gene Influenza H5/H7 Triplex kit (IDvet).

Samples with inconclusive results were tested twice on eggs. Hemagglutination assay was performed and HA-positive allantoic fluids were tested by rRT-qPCR for the H5 subtype to assess the presence of viral RNA.

Latent-Class Modeling

The analytical approach that was used modeled the cross-detection of farms whose true epidemiologic status (presence or absence of HPAI virus) was assessed using 4 different imperfect observation processes (based on 4 different sample types) of unknown sensitivity (defined as the probability of detecting the virus if it is present in the farm) and specificity (defined as the probability of not detecting the virus if it is absent from the farm). For each group of farms (clinically affected or not), the observed frequency of the $2^4 = 16$ different combinations of test results was assumed to have been distributed according to a multinomial distribution of parameters, n = 16 clinically affected farms (48 non-clinically affected farms) and 9 probabilities expressed as a combination of the proportion of infected farms and the sensitivity and specificity of each of the 4 sensitivity and specificity parameters. The analyses were performed in a Bayesian framework by using WinBUGS software (*2*) embedded in R software (*3*) by the R2WinBUGS library (*4*). For all sensitivity parameters, we assumed Uniform (0–1) as prior distributions. Because rRT-qPCR testing was considered to be highly specific (i.e., uncontaminated samples are very likely to test negative), Sp_{i_sample} parameters were assigned a β prior distribution defined such that its 5th percentile was equal to 90%, and its median to 98%. Given the high level of suspicion in clinically-affected flocks, we assumed a Beta (10–1) as a prior distribution for the proportion of infected flocks among clinically affected flocks. For the non-clinically affected flocks, we assumed a Uniform (0–1) prior distribution for the proportion of infected flocks. We ran 2 simulation chains of 100,000 iterations whose convergence and mixing were assessed by checking the trace plots for all monitored parameters and calculating the Gelman-Rubin convergence statistics (*5*). The first 5,000 iterations were discarded to allow for burn-in of the chains and the chains were thinned, taking every 100th sample to reduce autocorrelation among the samples.

Statistical Analysis

Differences in C_t distribution based on the sampling strategy were investigated by using a pairwise Wilcoxon test. For the calculation, negative C_t were associated to a C_t value of 40 and the mean C_t from wipes and swabs was calculated for each poultry house.

References:

1. Raynor PC, Adesina A, Aboubakr HA, Yang M, Torremorell M, Goyal SM. Comparison of samplers collecting airborne influenza viruses: 1. primarily impingers and cyclones. PLoS One. 2021;16:e0244977. PubMed https://doi.org/10.1371/journal.pone.0244977

2. Lunn DJ, Thomas A, Best N, Spiegelhalter D. WinBUGS—a Bayesian modelling framework: concepts, structure, and extensibility. Stat Comput. 2000;10:325–37. https://doi.org/10.1023/A:1008929526011

3. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2014.

4. Sturtz S, Ligges U, Gelman A. R2WinBUGS: a package for running WinBUGS from R. J Stat Softw. 2005;12:1–16.

5. Gelman A, Carlin JB, Stern HS, Rubin DB. Bayesian data analysis. CRC Press: Boca Raton, Florida; 2004.

Appendix Table 1. Cycle threshold values for tracheal swab and environmental samples collected on 63 poultry houses and tested for highly pathogenic avian influenza A(H5N8), France, December 2020–April 2021*

ID		Clinical	Wipes		Tracheal swab sample pools				Aerosol sampling†			
										NIOSH BC 251‡		
no.	Species	signs	Walls	Feeder	1	2	3	4	Coriolis	1	2	3
1	MD	Y	28.3	25.2	23.5	19.5	25.2	22.4	ND	ND	ND	ND
2	G	Y	29.29	27.91	25.2	26.7	26.3	27.1	ND	ND	ND	ND
3	MD	N	28.51	–	23.85	26.92	26.12	29.49	ND	ND	ND	ND
4	C	Y	29.44	29.17	25.26	25.51	23.31	24.96	ND	ND	ND	ND
5	MD	Y	26.81	23.67	19.76	24.98	25.30	24.93	23.39	26.82	30.56	33.76
6	MD	N	31.19	27.85	19.15	22.17	17.69	22.74	ND	ND	ND	ND
7	MD	N	25.1	23.71	20.44	21.86	20.82	21.13	ND	ND	ND	ND
8	MD	N	–	–	–	–	–	–	ND	ND	ND	ND
9	MD	N	–	–	–	–	–	–	34	36.06	–	–
10	C	Y	36.27	ND	–	24.78	24.51	20.02	32.67	35.81	36.22	–
11	MD	N	27.47	25.84	25.22	24.47	22.98	27.78	31.99	34	–	–
12	MD	Y	27.53	27.02	22.99	25.95	19.84	20.08	31.63	32.44	–	–
13	Q	N	–	–	–	–	–	–	–	–	–	–
14	Q	Y	33.77	34.63	30.15	29.71	24.7	27.22	30.18	36.11	–	–
15	MusD	N	25.2	23.53	24.14	24.69	24.88	29.34	ND	ND	ND	ND
16	MusD	N	25.35	24.91	34.19	27.65	32.82	33.29	ND	ND	ND	ND
17	MD	N	25.83	–	–	–	–	–	ND	ND	ND	ND
18	MD	N	–	–	–	–	–	–	ND	ND	ND	ND
19	C	N	–	–	–	–	–	–	–	–	–	–
20	C	Y	30.19	27.32	24.55	20.18	19.53	18.69	27.92	33.66	–	–
21	MD	N	34.04	28.69	26.48	21.76	25.76	21.03	ND	ND	ND	ND
22	PD	N	32.78	31.07	–	–	–	–	ND	ND	ND	ND
23	MD	Y	28.24	25.81	22.86	20.86	22.07	22.05	ND	ND	ND	ND
24	C	N	–	33.73	–	–	–	–	ND	ND	ND	ND
25	PD	N	32.05	31.15	–	36.11	–	–	31.56	–	–	–
26	MD	N	25.48	25.08	20.7	27.85	25.54	24.4	30.54	33.64	36	35.99
27	MD	N	26.44	ND	22.56	23.83	24.1	22.56	ND	ND	ND	ND
28	MD	Y	24.55	27.28	29.83	28.11	25.8	–	ND	ND	ND	ND
29	MD	N	30.14	27.43	21.92	19.02	19.58	20.96	31.23	27.84	32.36	36.3
30	MD	Y	28.35	29.51	18.86	23.15	17.87	27.02	24.85	25.81	33.13	–
31	PD	N	30.9	33.76	–	36.13	36.02	35.95	33	–	–	–
32	PD	N	–	–	–	–	–	–	ND	ND	ND	ND
33	MD	N	25.03	22.32	22.95	25.11	22.85	25.02	ND	ND	ND	ND
34	MD	N	22.99	24.23	19.94	21.22	18.09	20.63	26.9	23.75	18.63	–

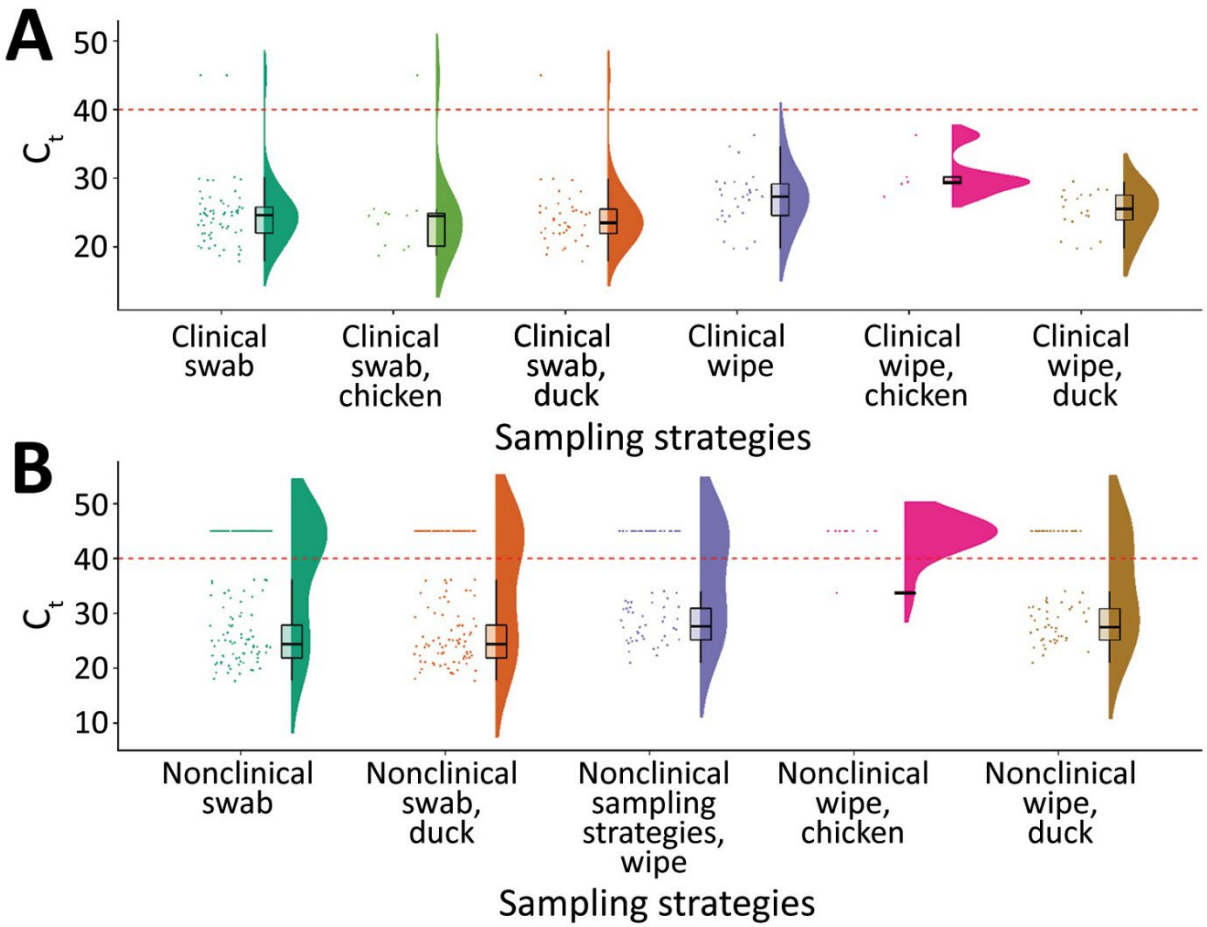
ID no.	Species	Clinical signs	Wipes		Tracheal swab sample pools				Aerosol sampling†			
			Walls	Feeder	1	2	3	4	Coriolis	NIOSH BC 251‡ 1	2	3
35	MD	Y	20.73	19.74	25.64	24.87	29.91	23.83	23.26	24.08	29.49	–
36	MD	Y	20.73	19.74	26.29	23.16	28.06	25.06	ND	ND	ND	ND
37	MD	N	31.97	30.28	27.74	–	24.46	30.03	27.52	33.01	–	36.21
38	MD	N	26.69	24.6	25.05	19.84	23.97	24.01	ND	ND	ND	ND
39	MD	N	–	27.07	21.09	19.22	22.68	19.68	ND	ND	ND	ND
40	MD	Y	24.94	24.46	21.9	24.1	29.7	21.2	ND	ND	ND	ND
41	MD	N	–	–	–	–	–	–	33.08	–	–	–
42	MD	N	–	30.77	–	–	–	–	ND	ND	ND	ND
43	MD	Y	27.59	23.99	21.71	21.98	22.65	24.72	22.15	25.55	–	35.87
44	MD	N	–	–	–	–	–	–	ND	ND	ND	ND
45	MD	N	–	–	–	–	–	–	ND	ND	ND	ND
46	MD	N	–	–	–	–	–	–	ND	ND	ND	ND
47	MD	N	–	–	–	–	–	–	ND	ND	ND	ND
48	MD	N	–	–	–	–	–	–	ND	ND	ND	ND
49	PD	N	32.3	ND	32.14	33.02	35.53	–	ND	ND	ND	ND
50	PD	N	30.8	27.3	23.9	25.3	29	29.7	ND	ND	ND	ND
51	PD	N	–	32	–	–	–	–	ND	ND	ND	ND
52	PD	N	29	21	26.2	22.3	22.3	31.1	ND	ND	ND	ND
53	PD	N	29.5	29.3	34.1	33.7	34.2	–	ND	ND	ND	ND
54	C	N	–	ND	–	–	–	–	ND	ND	ND	ND
55	C	N	–	–	–	–	–	–	ND	ND	ND	ND
56	C	N	–	ND	–	–	–	–	ND	ND	ND	ND
57	C	N	–	ND	–	–	–	–	ND	ND	ND	ND
58	C	N	–	ND	–	–	–	–	ND	ND	ND	ND
59	C	N	–	ND	–	–	–	–	ND	ND	ND	ND
60	C	N	–	ND	–	–	–	–	ND	ND	ND	ND
61	MD	N	–	ND	–	–	–	–	ND	ND	ND	ND
62	MD	N	–	ND	–	–	–	–	ND	ND	ND	ND
63	MD	N	–	ND	–	–	–	–	ND	ND	ND	ND

*C, chicken; C_t, cycle threshold; G, goose; ID, identification; MD, mule duck (a hybrid Muscovy/Peking); MusD, Muscovy duck; PD, Peking duck, Q, quail; ND, not done; NIOSH, National Institute for Occupational Safety and Health; –, no C_t value detected.
†Aerosol sampling was performed on 19 farms. Coriolis Compact (Bertin Instruments, https://www.bertin-instruments.com) and NIOSH BC 251 (National Institute for Occupational Safety and Health, https://www.cdc.gov/niosh) instruments were used.
‡NIOSH BC 251 sampling device has 3 fractions for different particle sizes; fraction 1 for >4 µm, 2 for 1–4 µm, and 3 for <1 µm.

Appendix Table 2. Official notification status of the animal houses included in a study for detection of highly pathogenic avian influenza A(H5N8) in dust from poultry farms, France, December 2020–April 2021*

Animal houses	Notification	House no.
HPAI-positive animal houses	Officially notified as outbreak	1, 2, 4, 5, 7, 10, 14, 20, 21, 23, 24, 26, 27, 28, 30, 31, 33, 34, 35, 36, 40, 43
	Official detection negative	NA
	Official detection not done	NA
Suspected animal houses adjacent to HPAI-positive poultry house	Officially notified as outbreak	49, 50, 51, 52, 53
	Official detection negative	19, 31, 41, 42
	Official detection not done	3, 6, 13, 15, 16, 22, 25, 29, 37, 38, 39, 44, 45, 46
Suspected animal houses epidemiologically related to HPAI-positive poultry house	Officially notified as outbreak	9, 11, 12
	Official detection negative	8, 47, 48, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63
	Official detection not done	17, 18

*HPAI, highly pathogenic avian influenza; NA, not applicable.



Appendix Figure. Half-violin, scatter, and box plots for C_t values of highly pathogenic avian influenza A(H5N8) virus detected from tracheal swab and environmental samples collected on poultry farms, France, December 2020–April 2021. C_t values are from results of real-time reverse transcription quantitative PCR on samples. A) Samples from 10 duck and 3 chicken houses where the animals had clinical signs of HPAI; and global results of 15 C_t values provide distribution comparison. B) Samples from 38 duck and 9 chicken houses where animals did not have clinical signs of HPAI; and global results of 48 C_t values provide distribution. Each dot indicates a C_t value from 1 wipe sample or 1 pool of 5 tracheal swab samples. Half-violins show distribution of C_t values for each sample type. Boxes show 95% CI for C_t values; horizontal lines in boxes indicate mean, vertical lines from boxes SD. Red dashed horizontal lines indicate C_t 40, the cutoff value for negative results. A pairwise Wilcoxon statistical test was used on the mean C_t values for wipes and swabs for each sampling strategy and animal house status. No statistically significant differences were found.