

Bioaerosol Sampling to Detect Avian Influenza Virus in Hanoi's Largest Live Poultry Market

Vuong N. Bui,¹ Tham T. Nguyen,² Hung Nguyen-Viet,^{3,4} Anh N. Bui,¹ Katie A. McCallion,⁵ Hu Suk Lee,³ Son T. Than,¹ Kristen K. Coleman,² and Gregory C. Gray^{2,6,7}

¹Virology Department, National Institute of Veterinary Research, Hanoi, Vietnam; ²Program in Emerging Infectious Diseases, Duke-NUS Medical School, Singapore; ³International Livestock Research Institute, Hanoi, Vietnam, and ⁴Center for Public Health and Ecosystem Research, Hanoi University of Public Health, Vietnam; ⁵College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, and ⁶Division of Infectious Diseases, Global Health Institute, and Nicholas School of the Environment, Duke University, Durham, North Carolina; and ⁷Global Health Research Center, Duke-Kunshan University, China

Background. Newly emergent and virulent strains of H7N9 avian influenza virus are rapidly spreading in China and threaten to invade Vietnam. We sought to introduce aerosol sampling for avian influenza viruses in Vietnam.

Methods. During October 2017, National Institute for Occupational Safety and Health 2-stage aerosol samplers were assembled on a tripod and run for 4 hours. Concomitantly, up to 20 oropharyngeal (OP) swab samples were collected from chickens and ducks distanced at 0.2–1.5 m from each sampler.

Results. The 3 weeks of sampling yielded 30 aerosol samples that were 90% positive for influenza A, by quantitative reverse-transcription polymerase chain reaction, and 116 OP swab sample pools (5 samples per pool) that were 47% positive. Egg cultures yielded 1 influenza A virus (not H5 or H7) from aerosol and 25 influenza A viruses from OP swab sample pools (5 were H5 positive). The association between positive sample types (over time and position) was strong, with 91.7% of positive OP pooled swab samples confirmed by positive aerosol samples and 81% of influenza A positive aerosol samples confirmed by positive OP swab samples.

Conclusions. We posit that aerosol sampling might be used for early warning screening of poultry markets for novel influenza virus detection, such as H7N9. Markets with positive aerosol samples might be followed up with more focused individual bird or cage swabbing, and back-tracing could be performed later to locate specific farms harboring novel virus. Culling birds in such farms could reduce highly pathogenic avian influenza virus spread among poultry and humans.

Keywords. avian influenza; influenza A virus; Vietnam; poultry; epidemiology.

In recent years, avian influenza virus outbreaks have caused a massive number of poultry deaths, resulting in the destruction of poultry markets and farms in multiple world regions [1–4]. Monitoring and controlling such outbreaks among poultry is important for public safety, because human infections can occur through direct contact with infected poultry and their surrounding environments. For example, H5N1 avian influenza virus outbreaks resulted in 26 human infections and 14 deaths in Cambodia in 2013, and 173 human infections and 53 deaths in Egypt from 2014 to 2015 [5]. More recently, from October 2016 to 28 March 2018, the H7N9 avian influenza virus outbreak resulted in 822 confirmed human infections and at least 307 human deaths [6]. Furthermore, the H7N9 virus has spread to western China (Guangxi Province), and a highly pathogenic strain has emerged [7], which scientists anticipate will soon

breach the China-Vietnam border through formal and informal live poultry trade [8].

To mitigate the further spread of H7N9, Vietnam's Ministry of Health's General Department of Preventive Medicine has ordered public safety dispatchers to halt the smuggling of poultry and poultry products from the Chinese border into Vietnam. Health workers in bordering provinces have also been trained in the surveillance, diagnostic testing, and treatment for potential outbreaks [9]. However, despite diligent preparations, Vietnam's surveillance system is spotty, lacking a quick and effective tool to detect the incursion of viruses in large-scale poultry markets.

In a pilot effort to enhance avian influenza virus surveillance, our team employed a bioaerosol sampling method using National Institute for Occupational Safety and Health (NIOSH) 2-stage aerosol samplers [10–12] to monitor for and capture live avian influenza H5N1 and H7N9 viruses from the largest live poultry market in Hanoi, Vietnam.

MATERIALS AND METHODS

Study Site

Our study took place during the first 3 weeks of October 2017 at Ha Vi Market (Supplementary Figure 1), the largest live wholesale poultry market in Hanoi, Vietnam. It is estimated that

Received 23 April 2018; editorial decision 29 June 2018; accepted 11 August 2018; published online August 31, 2018.

Correspondence: G. C. Gray, Division of Infectious Diseases, Duke University Medical Center, Duke University, Box 102359, Durham, NC 27710 (gregory.gray@duke.edu).

Clinical Infectious Diseases® 2019;68(6):972–5

© The Author(s) 2018. Published by Oxford University Press for the Infectious Diseases Society of America. All rights reserved. For permissions, e-mail: journals.permissions@oup.com. DOI: 10.1093/cid/ciy583

80 000–100 000 live birds are sold most every day of the week in this market, which operates over an area of 20 000 square meters with 200 registered shops.

Bioaerosol Sampling

Three NIOSH BC 251 aerosol samplers were assembled on a tripod and positioned 0.5 m from the ground and 0.2–1.5 m from live bird cages on 10 days during the 3-week period. Each NIOSH sampler was connected to an AirChek TOUCH Sample Pump (SKC) set at a flow rate of 5 L/min for 4 hours, filtering a total of 1200 L of air through each sampler. Aerosols were captured in a 15-mL Falcon tube, 1.5-mL centrifuge tube, and a polytetrafluoroethylene filter cassette attached to each NIOSH sampler. After each sampling session, sample tubes and filters were transported on ice to the laboratory, where they were quickly processed and preserved at -80°C .

Poultry Swabbing

During each of the 10 sampling days, 15–20 oropharyngeal (OP) swab samples were collected from chickens and ducks 0.2–1.5 m from each of the 3 NIOSH samplers (Supplementary Figure 2). After collection, OP swab samples were pooled (5 samples in a single 2.5-mL tube of viral transport medium). OP swab sample pools were kept on ice until being transported back to the laboratory, where they were processed and preserved at -80°C . The National Institute of Veterinary Research required no special ethical approval to conduct this routinely performed poultry swab sampling.

Sample Processing

Aerosol Samples

In the laboratory, the 15-mL Falcon tubes and 1.5-mL centrifuge tubes were detached from the NIOSH samplers, after which 2 mL of sterile virus collection medium (phosphate-buffered saline with 0.5% wt/vol bovine serum albumin fraction V) was added to each 15-mL centrifuge tube, and 1 mL of virus collection medium was added to each 1.5-mL centrifuge tube. Sampler tubes were then vortexed both upright and inverted, to ensure that the tops of the tubes were washed thoroughly. The vortexed bovine serum albumin solutions were then transferred to 2.0-mL cryovial tubes.

Filter cassettes were then removed from the NIOSH samplers and each polytetrafluoroethylene filter was carefully transferred to the bottom of a 500-mL Falcon tube and vortexed for 15 seconds while dry. Next, 1 mL of virus collection medium was added to each 50-mL tube and vortexed twice before removing and discarding the filter. The vortexed sample solutions in the 50-mL tubes were then transferred to the 2.0-mL cryovial tubes containing the sample solutions from the 1.5-mL centrifuge tubes previously detached from the NIOSH samplers, yielding a 2-mL combined sample tube. All aerosol sample solutions were stored at -80°C until they were used for further analyses.

Swab Samples

Tubes containing pooled OP swab samples were vortexed twice at medium speed before removal and discarding of the swabs. The remaining solution was transferred to cryovial tubes and stored at -80°C until used for further analyses.

Molecular Assays

Using the QIAamp Viral RNA Mini Kit (Qiagen), total RNA was extracted from 140 μL of each aerosol and pooled OP swab sample and eluted in 60 μL of elution buffer. Using an IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) and a Superscript III Platinum One-step RT-PCR kit (Thermo fisher Scientific), extracted sample RNA was tested for the presence of avian influenza virus by means of real-time reverse-transcription polymerase chain reaction (RT-PCR) targeting the influenza A virus M gene [13]. Cycling conditions used were as follows: 50°C for 30 minutes, 95°C for 15 minutes, and 40 cycles of 95°C for 10 seconds and 58°C for 50 seconds. The primers and probe used were as follows: forward primer, 5'-GAC CRA TCC TGT CAC CTC TGA C-3'; reverse primer, 5'-AGG GCA TTY TGG ACA AAK CGT CTA-3'; and probe, FAM-TGC AGT CCT CGC TCA CTG GGC ACG-BHQ1. Any sample with a cycle threshold (Ct) value <40 was considered positive for the M gene. A single aerosol sample was considered positive if either the 15-mL tube sample or the pooled 1.5-mL tube and filter sample was positive. Positive samples were subtyped for A/H5 and A/H7, using primer-probe sets described elsewhere [14, 15].

Viral Isolation

A 0.1-mL volume of each of the 30 aerosol samples and each of the 116 pooled OP swab samples was inoculated in triplicate into the allantoic cavity of 9–10-day-old embryonated chicken eggs (438 eggs total). Eggs were incubated at 37°C for 3 days and chilled at 4°C for ≥ 24 hours. Allantoic fluid from each egg was tested with a hemagglutination (HA) assay described in the World Health Organization manual [16].

Data Analysis

The McNemar test was used to evaluate the association of results between aerosol and swab samples. Differences were considered statistically significant at $P < .05$. This test was conducted using Stata software (version 14.0; StataCorp). QGIS software (version 3.0.1; Quantum GIS development Team 2018) was used to generate the map.

RESULTS

During the first 3 weeks of October 2017, a total of 30 aerosol samples and 580 poultry OP swab samples were collected. Twenty-seven (90%; 95% confidence interval [CI], 73.5%–97.9%) of the aerosol samples and 54 (47%; 37.2%–56.0%) of the 116 pools of poultry OP swab samples were positive for

influenza A as demonstrated by real-time RT-PCR. None of the influenza A–positive aerosol samples were positive for H5 and H7 genes. The mean Ct values for influenza A–positive aerosol and pooled OP swab samples were 35.04 and 28.74, respectively.

After egg culture, HA test results suggested viral growth in 1 (3%) of the aerosol samples and 43 (37%) of the pooled poultry OP swab samples. The aerosol sample with evidence of a pathogen in egg culture had molecular evidence of influenza A, but the H5 and H7 assay results were negative. Among the 43 egg cultures with suggested pathogen growth, molecular assays revealed that 25 (58%) were influenza A positive, with 5 positive for H5 and none positive for H7.

Aerosol Pooled OP Swab Sample Comparison

We compared the aerosol and pooled OP swab samples that were collected at the same time in the same areas of the bird market (Table 1). When assay results were positive by either method, association statistics were remarkably strong. The percentage of positive agreement between 2 sampling methods was 91.7% (95% CI, 73%–99%) when we used swab sampling as the reference test. Alternatively, when we used aerosol sampling as the reference test, the percentage of positive agreement was 81% (95% CI, 62%–94%). The overall agreement was 77% (95% CI, 58%–90%). The McNemar test ($P = .26$) suggested no statistically significant difference between sampling methods.

DISCUSSION

We conducted a pilot bioaerosol sampling study at a large live bird market in Hanoi with a goal of screening for avian influenza A viruses among live poultry. A total of 30 aerosol and 116 poultry OP swab samples were collected. Our percentage of positive aerosol samples (90%) for influenza A virus was high compared with results in previous aerosol studies [17, 18]. We had a strong agreement between positive aerosol and swab samples, supporting the premise that noninvasive aerosol sampling might be an efficient and low-cost means of screening large populations of poultry for avian influenza virus. In particular, aerosol sampling might be effective in detecting incursions of novel viruses, such as H7N9.

Aerosol sampling is an attractive surveillance tool, because it does not involve the handling of birds and requires no animal ethical committee approval. In addition, aerosol equipment is compact, portable, and convenient and can cover relatively large geographic areas, with studies in livestock agriculture detecting novel virus incursions 2–16 km from the source [19]. In contrast, poultry throat or cloacal swab sample surveillance studies require animal ethical approvals and are often met with resistance by poultry owners.

The current study had a number of limitations. The quantitative RT-PCR–positive aerosol samples had a higher mean Ct value than the quantitative RT-PCR–positive pooled swab samples (35.04 and 28.74, respectively), suggesting more virus particles in the swab samples. This would explain the recovery of only 1 live influenza A virus from 30 aerosol samples, compared with 25 live influenza A viruses from 116 pooled OP samples. The eggs used in this study were not certified commercially as pathogen free. Hence, other pathogens in the eggs or in the field samples may have confounded HA assay results. For instance, in another unpublished study, we have found Newcastle disease virus to cause HA-positive egg cultures.

Furthermore, not all aerosol samplers perform equally. The NIOSH samplers used in this study, though small and portable, may not detect pathogens at distances equal to those enabled by larger, more cumbersome aerosol samplers [19]. Hence, it seems likely that controlled comparison experiments with multiple samplers will be required to determine the optimal aerosol sampling strategies for such surveillance. Furthermore, our pilot study was small, with 90% of aerosol samples testing positive, limiting our ability to make conclusions comparing the specificity of aerosol and poultry swab sampling. Finally, because our collaborators at the National Institute of Veterinary Research did not have the ability to immediately sequence type the viruses, we do not know with absolute certainty that the aerosol detections represent influenza A RNA from the birds.

Even with these limitations, it seems clear that periodic aerosol sampling might be used to economically screen large bird markets for novel pathogens. Positive aerosol samples are very likely to reflect the presence of influenza A virus in a market.

Table 1. Comparison of Aerosol Sampling Assays With Pooled OP Swab Sample Assays for Real-time RT-PCR Evidence of Influenza A Virus^a

Aerosol Samples	Pooled Poultry OP Swab Samples Collected Near Aerosol Sample ^b		Agreement (95% CI), %		
	Positive	Negative	Positive	Negative	Overall
Positive	22	5	91.7 (73–99)	16.7 (0.4–64.1)	76.7 (57.7–90)
Negative	2	1			
Total	24	6			

Abbreviations: CI, confidence interval; OP, oropharyngeal; RT-PCR, reverse-transcription polymerase chain reaction.

^aAerosol and swab samples were obtained at the same time and in the same location in the Ha Vi live bird market.

^bSamples obtained 0.2–1.5 m from the aerosol collection point.

Such positive aerosol findings might be followed up with poultry or cage swab sampling in aerosol-positive stall areas. Positive poultry or cage samples might then be back-traced to specific farms.

In conclusion, we believe this to be the first study to use bioaerosol sampling for virus surveillance in a bird market in Vietnam. Our study results suggest that bioaerosol sampling may offer a new, noninvasive tool for animal or human pathogen surveillance.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. We are indebted to administrators and staff at the sampling site, Ha Vi market, for granting permission to perform the field collections; staff at National Institute of Veterinary Research for their contributions to sample collection and laboratory assays; Bill Lindsley of the NIOSH for aerosol sampling support; Pawin Padungtod and Leo Loth of the Food and Agriculture Organization of the United Nations; and Jeffrey W McFarland, MD, and Anthony Mounts of the US Centers for Disease Control and Prevention office in Vietnam for advice regarding avian influenza prevalence in Vietnam.

Financial support. This work was supported by the Consultative Group for International Agricultural Research (CGIAR) research programs on Agriculture for Nutrition and Health and Climate Change, Agriculture and Food Security and the by Laboratory of One Health Research at Duke-NUS Medical School, Singapore.

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Bi Y, Zhang Z, Liu W, et al. Highly pathogenic avian influenza A(H5N1) virus struck migratory birds in China in 2015. *Sci Rep* **2015**; 5:12986.
2. Haider N, Sturm-Ramirez K, Khan SU, et al. Unusually high mortality in waterfowl caused by highly pathogenic avian influenza A(H5N1) in Bangladesh. *Transbound Emerg Dis* **2017**; 64:144–56.

3. Monne I, Meseko C, Joannis T, et al. Highly pathogenic avian influenza A(H5N1) virus in poultry, Nigeria, 2015. *Emerg Infect Dis* **2015**; 21:1275–7.
4. Horm SV, Tarantola A, Rith S, et al. Intense circulation of A/H5N1 and other avian influenza viruses in Cambodian live-bird markets with serological evidence of sub-clinical human infections. *Emerg Microbes Infect* **2016**; 5:e70.
5. World Health Organization. Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO, 2003-2018 2018. Available at: http://www.who.int/influenza/human_animal_interface/2018_03_02_tableH5N1.pdf?ua=1. Accessed 6 April 2018.
6. Food and Agriculture Organization (FAO) of the United Nations. H7N9 situation update. Available at: http://www.fao.org/ag/againfo/programmes/en/empres/h7n9/wave_6/Situation_update_2018_03_28.html. Accessed 6 April 2018.
7. Qi W, Jia W, Liu D, et al. Emergence and adaptation of a novel highly pathogenic H7N9 influenza virus in birds and humans from a 2013 human-infecting low-pathogenic ancestor. *J Virol* **2018**; 92. doi:10.1128/JVI.00921-17.
8. Armed Forces Health Surveillance Branch H7N9 surveillance summary 2017. Available at: <http://www.fao.org/vietnam/news/detail-events/en/c/1128685/>. Accessed August 3 2018.
9. Coston M. Avian flu diary: Vietnam girds against H7N9 2017. Available at: <http://afludairy.blogspot.sg/2017/03/vietnam-girds-against-h7n9.html>. Accessed 6 April 2018.
10. Ahrenholtz S, Brueck SE, De Perio MA, Blachere FM, Lindsley WG. Environmental assessment for the presence of influenza viruses (2009 pandemic influenza A H1N1 and seasonal) in dental practices—Ohio. Cincinnati, Oh: NIOSH, **2011**.
11. Blachere FM, Cao G, Lindsley WG, Noti JD, Beezhold DH. Enhanced detection of infectious airborne influenza virus. *J Virol Methods* **2011**; 176:120–4.
12. Blachere FM, Lindsley WG, Pearce TA, et al. Measurement of airborne influenza virus in a hospital emergency department. *Clin Infect Dis* **2009**; 48:438–40.
13. The WHO Collaborating Center for influenza at the CDC Atlanta. CDC protocol of realtime RTPCR for influenza A (H1N1). Available at: http://www.who.int/csr/resources/publications/swineflu/CDCRealtimeRTPCR_SwineH1Assay-2009_20090430.pdf. Accessed 6 April 2018.
14. Avian Influenza Community Reference Laboratory. Eurasian H5 avian influenza realtime PCR. Available at: <https://science.vla.gov.uk/flu-lab-net/docs/pub-protocol-ai-vi492.pdf>. Accessed 6 April 2018.
15. Food and Agriculture Organization of the United Nations. H7-specific real-time RT-prediction of novel H7N9. Available at: http://www.fao.org/ag/againfo/programmes/en/empres/H7N9/docs/diagnostic_table.pdf. Accessed 6 April 2018.
16. World Health Organization. Manual for the laboratory diagnosis and virological surveillance of influenza. Available at: http://www.who.int/influenza/gisrs_laboratory/manual_diagnosis_surveillance_influenza/en/. Accessed 6 April 2018.
17. Wu Y, Shi W, Lin J, et al. Aerosolized avian influenza A (H5N6) virus isolated from a live poultry market, China. *J Infect* **2017**; 74:89–91.
18. Kang N, Chen M, Bi FY, Chen MM, Tan Y. First positive detection of H9 subtype of avian influenza virus nucleic acid in aerosol samples from live poultry markets in Guangxi, South of China. *Chin Med J (Engl)* **2016**; 129:1371–3.
19. Anderson BD, Lednicky JA, Torremorell M, Gray GC. The use of bioaerosol sampling for airborne virus surveillance in swine production facilities: a mini review. *Front Vet Sci* **2017**; 4:121.