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BRIEF REPORT

Bioaerosol Sampling at a Live Animal Market in Kunshan, China: A Noninvasive Approach for Detecting Emergent Viruses

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Emerging zoonotic viruses have led to considerable human and animal morbidity and mortality across China. Examples include the severe acute respiratory syndrome coronavirus (SARS-CoV), avian strains of influenza A virus (eg, H5N1, H7N9, and H9N2), and, most recently, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1–4]. China is considered an ideal environment for novel respiratory virus emergence, as dense populations of humans and livestock live in close proximity [5]. The SARS-CoV-2 pandemic illustrates how quickly a local emerging zoonotic virus epidemic in humans can move throughout the world [6]. Previous human infections with avian influenza viruses (AIVs), including H7N9, H5N1, H5N6, and H9N2 [1, 3, 7] in China have raised pandemic concerns. Many of these human infections were associated with exposures at live bird markets (LBMs) [1, 2]. Although the provincial and local government authorities in China have sometimes closed the LBMs after these human outbreaks, live poultry trading has gradually been permitted to return, as many Chinese people prefer fresh poultry meat over processed poultry meat [8]. Although large cities in China have largely moved to processed poultry, much of the rest of China continues to sanction LBM activity. Chinese LBMs often have multiple species of live poultry for sale, including chickens, ducks, quails, geese, and pigeons. These different species of live poultry are often mixed in cages and have beak-to-beak contact, which significantly increases the risk of AIV transmission across species. Additionally, poultry are often purchased and butchered on site, with little concern for potential human exposure or biosecurity. Such LBM conditions may pose a threat to public health.

In this pilot study, we sought to examine molecular evidence for influenza A, B, C, and D viruses in a single LBM located in Kunshan, China.

METHODS

Ethics Statement and Study Location

Bioaerosol samples were obtained from a small LBM (6 small open stalls and 1 poultry slaughter room) in Kunshan City, Jiangsu Province, China. Kunshan is located at the heart of the Yangtze River Delta region in Eastern China's Jiangsu province. Sampling was conducted from October to December 2018. As this study did not involve human sampling or handling animals, an ethical review was not required. The study was explained to the vendors as a graduate school research project, and they were assured they would not be identified. Each of the vendors gave verbal permission for the samplers to be periodically set up near their poultry stalls.

Sample Collection

Bioaerosol Sampling

This study was conducted in a small LBM in Kunshan City, China. Study personnel from Duke Kunshan University (DKU) traveled to this small LBM 2 to 4 times per week from October to December to collect aerosol samples using a National Institute of Occupational Safety and Health (NIOSH) 2-stage bioaerosol cyclone sampler connected to a SKC AirCheck Touch personal sampling pump (Cat#: 220-5000TC-K; SKC Inc., Eighty Four, PA, USA) [9]. During each visit, 2 NIOSH 2-stage aerosol samplers were placed in 2 locations in the LBM close to the poultry cages. The first sampler was set 0.5–1 m above the cages; the second was set ~1.3 m above the ground on a tripod and ~0.5 m away from the poultry cages (Supplementary Figure 1). Sampling, sample processing, molecular assays, viral culture, gene sequencing, and phylogenetic analysis were performed per previous reports (Supplementary Data).

RESULTS

Sixty-six (55.0%) of the 120 bioaerosol samples collected from October 2018 to December 2018 were positive for influenza

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A by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The mean Cq value for influenza A–positive bioaerosol samples was 34.7. Among these positive bioaerosol samples, 36 (54.5%) were from the first stage (particle diameter >4 μm), and 30 (45.5%) were retrieved from the combined samples of the second stage (particle diameter 1–4 μm) and the filter (particle diameter <1 μm) (Table 1). None of the bioaerosol samples were positive for influenza B, C, or D virus.

Among the 66 influenza A–positive samples, 9 samples (13.6%) were positive for the H7 subtype and 40 samples (60.6%) were positive for the H9 subtype by qRT-PCR. Samples with mixed H7 and H9 subtypes were also detected (Table 1). No samples were positive for the H5 subtype.

Sixty out of 66 positive samples had enough volume to attempt viral culture in eggs. Four H9N2 strains from 60 aerosol samples were successfully isolated in this study. The influenza A mean Cq value of these 4 specimens that yielded H9N2 viruses in viral culture was 9.9. All these strains were detected from the first stage of the NIOSH sampler with particles >4 μm, with an isolation success ratio of 4/36.

To understand the genetic relationships between these 4 isolates and previously published H9N2 viruses, phylogenetic comparisons were made examining both the viral HA and NA genes. The phylogenetic comparisons of 4 isolates' HA genes were made with 49 reference strains (Supplementary Table 1) of 3 distinct Eurasian avian H9N2 lineages, such as A/Chicken/Beijing/1/94-like (BJ/94-like), A/Duck/Hong Kong/Y439/97-like (Y-439 like), and A/Quail/Hong Kong/G1/97-like (G1-like). The HA genes from the 4 H9N2 isolates shared high nucleotide homology with each other (93.3%–98.6%) and were clustered with the A/Chicken/Zhejiang/HJ/2007 virus (G57 genotype), which was derived from the BJ/94-like lineage (Figure 1A). The results also demonstrated that the 4 isolates had a close relationship with chicken H9N2 isolates that were circulating in Jiangsu province between 2017 and 2018 (eg, A/Chicken/Jiangsu/J2463/2017 [H9N2], which shared 93.6%–99.3% identity scores and A/Chicken/Jiangsu/J2423/2018 [H9N2], which shared 93.2%–99.4% identity scores). The phylogenetic analysis of the NA nucleotide sequences showed that the 4 isolates shared high sequence identity (96.7%– 98.5%) and fell into the G57 NA genotype cluster (Figure 1B). These isolates were also found to have a close relationship with a chicken H9N2 isolate that was circulating in Shanghai in 2018 (A/Chicken/Shanghai/07/2018 [H9N2]), which shared 96.6%–98.1% sequence identity. In addition, the results of phylogenetic trees (Figure 1A and B) indicated that both HA and NA genes of 4 isolates belonged to a Eurasian avian linage and were from the genotype classified G57.

DISCUSSION

Mounting evidence suggests that aerosol transmission of influenza viruses is a possible mode of human-to-human transmission [10, 11], but studies focused on zoonotic influenza virus transmission at the human–animal interface are few. We conducted bioaerosol surveillance for influenza viruses at a small LBM in Kunshan City and found a high prevalence (55%) of influenza A–positive samples by qRT-PCR. This finding is consistent with previous similar aerosol studies [12, 13] in China. Remarkably, we also were able to isolate live virus in 4 specimens using a dry sampler without viral transport media, suggesting that a high quantity of viable virus particles were airborne. These findings strengthen arguments that aerosol transmission to birds and humans may be occurring in LBMs. Our data also support the position that bioaerosol sampling has great potential as a noninvasive surveillance tool for detecting aerosolized influenza A virus wherever humans and animals mix.

Our data also indicate that viral RNA and viable viruses were primarily detected in the first stage of the NIOSH sampler (particles size >4 μm). This finding is consistent with 2 previous studies: Zhou et al. (2016) [13] conducted a similar surveillance study using both the NIOSH sampler (BC251) and the Coriolis μ air sampler in 3 poultry markets in Guangzhou, China. In that study, influenza A–positive samples were detected at a rate of 87.5%–100% in the first stage of the sampler. Later, Zhou et al. (2018) [12] demonstrated that influenza virus–laden particles from donor ferrets were predominantly detected in particles >4 μm in diameter by using the NIOSH sampler. However, 30%

Table 1. Molecular Detections of Influenza A Virus From 120 Bioaerosol Samples Obtained During October—December 2018 From a Live Poultry Market, Kunshan City, Jiangsu Province, China

Bioaerosol Samples	No. (%) of Influenza A–Positive Samples by qRT-PCR	No. (%) of Isolates	HA Subtype of Influenza A–Positives Samples				
			H5, No. (%)	H7, No. (%)	H9, No. (%)	Both H7 and H9, No. (%)	Non-H5/H7/H9, No. (%)
Particles >4 μm (n = 60)	36 (54.6)	4 (11.1)	0	4 (11.1)	29 (80.6)	4 (11.1)	7 (19.4)
Particles ≤4 μm (n = 60)	30 (45.4)	0 (0)	0	5 (16.7)	11 (36.7)	2 (6.7)	16 (54.3)

Each hour-long bioaerosol run yielded 1 sample for particles >4 μm and 1 sample for particles ≤4 μm.

Abbreviation: qRT-PCR, quantitative reverse transcription polymerase chain reaction.

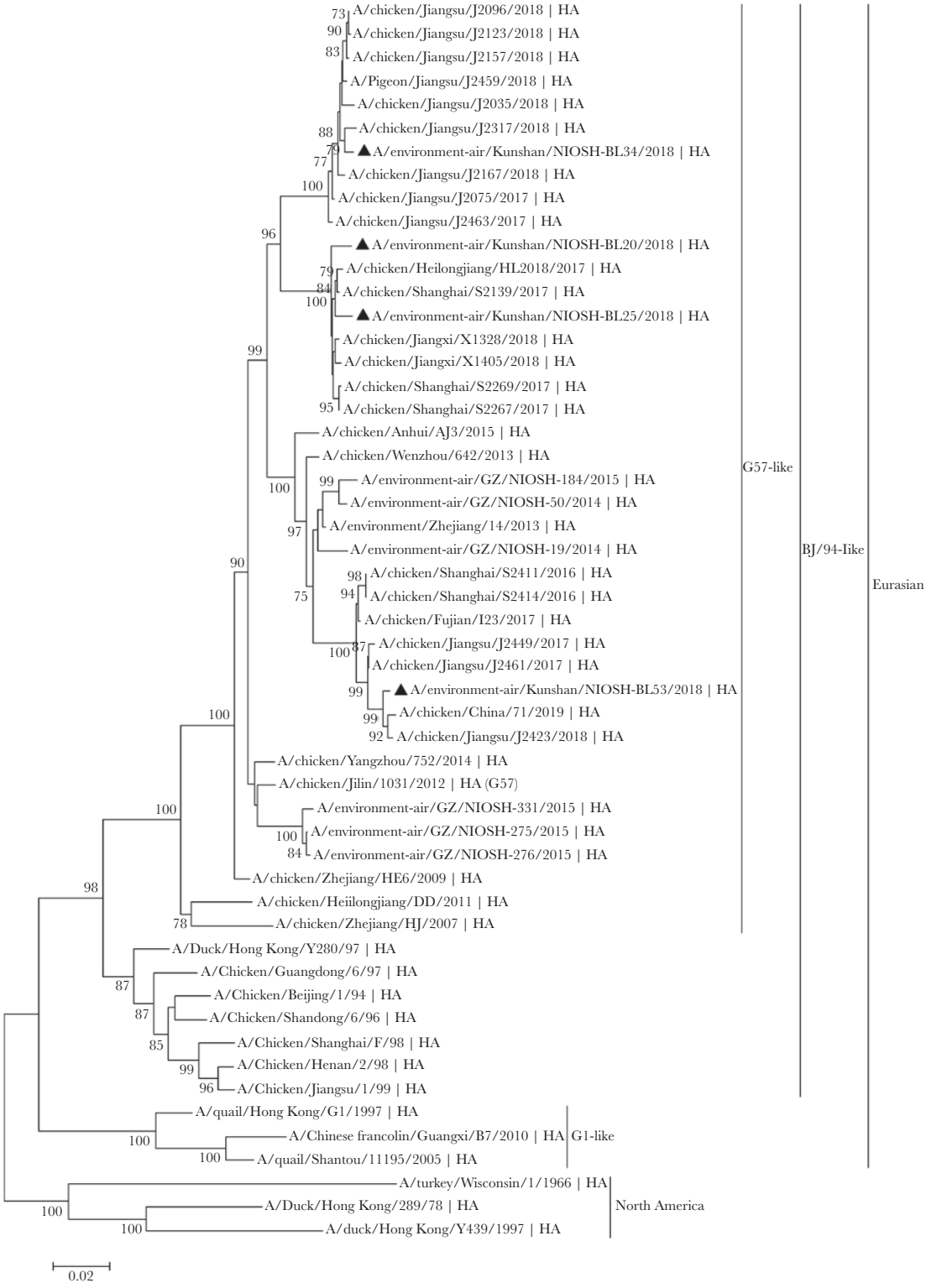

Figure 1. Phylogenetic trees of the hemagglutinin (HA) (A) and neuraminidase (NA) (B) genes of various influenza A H9N2 viruses. The H9N2 viruses detected at a live poultry market in Kunshan (Jiangsu Province), China, from October to December 2018 are marked with solid triangles. Other H9N2 viruses were gathered from numerous other publications (Supplementary Table 1). The phylogenetic tree was constructed with a neighbor-joining tree method and Kimura 2-parameter model using MEGA software, version 7 (https://www.megasoftware.net). Bootstrap values were calculated on 1000 replicates, and values <70% are not shown. Abbreviations: BJ/94-like:A(H9N2), A/Chicken/Beijing/1/94-like virus; G1-like:A(H9N2), A/quail/Hong Kong/G1/1997-like virus; G57-like:A(H9N2), A/Chicken/Zhejiang/HJ/2007-like virus; Y439-like:A(H9N2), A/duck/Hong Kong/Y439/1997-like virus.

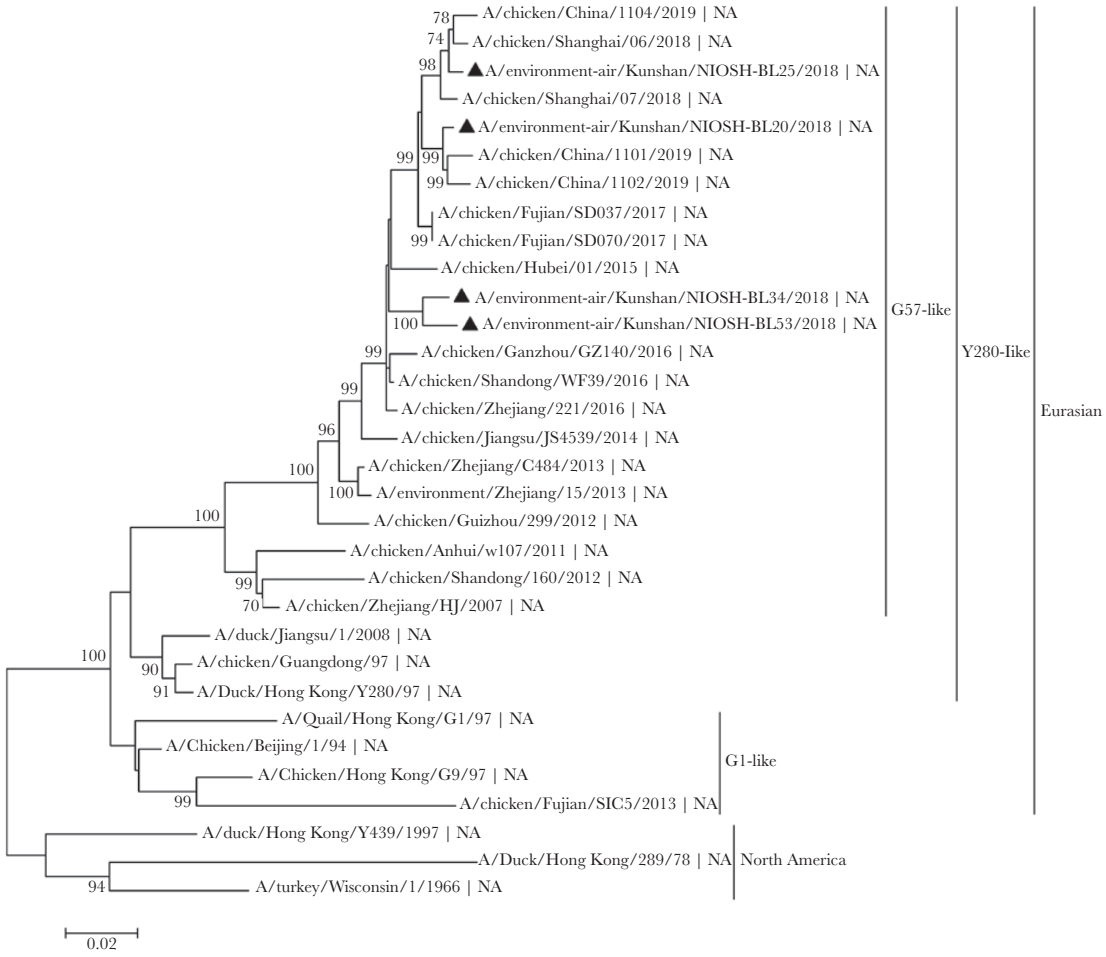

Figure 1. Continued.

of our positive samples were of smaller molecular size, suggesting that such smaller particles may circulate longer in the air and disperse over longer distances. Particles of such smaller size may be more likely to deposit in the lower respiratory tract, potentially causing lower respiratory track illness [11, 14]. As such, our detection of influenza A viruses in an LBM is significant, as many people (including the elderly and children) visit this LBM every day. Although they may not directly touch or process the poultry, these data suggest that proximity to bird cages could be a risk factor for avian influenza virus infection. Through observing LBM activities, we observe a number of ways AIVs could be aerosolized at the market: frequent live bird wing flapping movements within and outside of cages, frequent human handling of birds, movement of birds from cages to a slaughter room, use of defeathering machines, afternoon cage and slaughter room water wash downs, etc. More comprehensive environmental sampling might be employed to determine which specific activities in the live bird markets are aerosolizing viral particles and to try to identify interventions to mitigate these risks.

Of the 66 aerosol-positive samples for influenza A virus RNA by qRT-PCR, H9 was the most prevalent subtype, followed by the H7 subtype. Four H9N2 viruses were isolated among the 40 samples positive for the H9 subtype. We did not isolate any H7 influenza viruses. Phylogenetic analyses demonstrated that the HA gene sequences of all 4 isolates were homologous (93.3%–98.6%) and clustered with A/Chicken/Zhejiang/HJ/2007 virus (G57 genotype), suggesting that these isolates and the G57 ZJ/HJ/07 strain share a common ancestor. It is not surprising that these G57-like viruses were found in the LBM, as G57 strains have been reported as the most prevalent genotype circulating in chickens in China since 2013 [15]. Previous studies in Jiangsu province (where Kunshan is located) in 2013–2016 [16] and in Guangzhou between 2014 and 2015 [13] have provided molecular evidence that H9N2 subtypes are enzootic in poultry markets. Perez et al. [17] have also posited that H9N2 replicates more efficiently in the respiratory tract of land fowls (eg, chicken, quail, pheasant, chukar, and other minor domestic poultry), explaining this high prevalence.

The LBM in this study was a retail market, consisting of 6 stalls and 1 slaughter room. This market often had multiple species of live birds at a time, including aquatic birds (ducks and geese), land fowl (chickens, quails, and pigeons), and other

poultry (especially pheasants). Although aquatic birds and land fowl were generally kept in separate cages, the distance between cages was often <0.2 m. Previous studies have shown that aquatic birds may act a reservoir for various influenza virus subtypes, which can be transmitted to other avian species including humans. Although H9N2 infections in humans have caused only mild symptoms (eg, fever and coughing), it seems prudent, nevertheless, to warn poultry workers of their occupational exposures [18]. Additionally, although H9N2 viruses are considered to be "low pathogenic" to poultry [19], they have contributed through reassortment to the emergence of other avian influenza virus (AIV) subtypes. Specifically, H9N2 viruses are thought to have provided 6 internal genes to recently circulating H7N9 virus in China and to have donated partial internal genes to other AIV subtypes, such as H5N1, H5N6, and H10N8 viruses [15, 20]. Thus, this LBM's high prevalence of AIV and specifically of H7 and H9 strains could pose a threat both to domestic poultry flocks and to the public's health.

It seems clear that NIOSH 2-stage samplers in this and other poultry settings [13, 21, 22] have great potential value for future AIV surveillance work. Although they are dry samplers, which are not optimal for live virus collection compared with traditional surveillance tools (eg, poultry throat or cloacal swabs), the aerosol samplers are noninvasive, and their use does not require animal ethical approvals. Aerosol samplers also cover relatively large areas, and market vendors are more likely to permit this type of sampling, even when the devices are placed very close to their poultry cages.

The study was limited to 1 live bird market in 1 geographic area, and data should not be extrapolated elsewhere. We also are not sure if any of the poultry on sale had received influenza vaccines. This study was further limited in that we were not able to compare the molecular evidence of influenza viruses detected by bioaerosol samplers with traditional poultry sampling methods in the LBM. This study was also limited in that we could not associate our bioaerosol results to illnesses experienced by poultry vendors or LBM visitors.

Even with these limitations, our findings demonstrate the utility of employing aerosol sampling in LBMs. Aerosol sampling has an advantage in that it is a noninvasive approach and is generally accepted by animal vendors. Bioaerosol sampling may be first used as a screening tool, and then if evidence of a novel virus is found, it may be followed up with more traditional animal swabbing methods. Aerosol sampling can cover large market areas in an efficient manner. Considering the current SARS-CoV-2 pandemic, one wonders if aerosol sampling might be periodically employed in such live animal markets, along with systematic metagenomic analyses of the sampling products, to give early warning of emergence of a novel virus.

Supplementary Data

Supplementary materials are available at *Open Forum 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.

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