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Interpretation of molecular detection of avian influenza A virus in respiratory specimens collected from live bird market workers in Dhaka, Bangladesh: infection or contamination?

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ABSTRACT

Objectives: Interpreting real-time reverse transcription-polymerase chain reaction (rRT-PCR) results for human avian influenza A virus (AIV) detection in contaminated settings like live bird markets (LBMs) without serology or viral culture poses a challenge.
Methods: During February-March 2012 and November 2012-February 2013, we screened workers at nine LBMs in Dhaka, Bangladesh, to confirm molecular detections of AIV RNA in respiratory specimens with serology. We tested nasopharyngeal (NP) and throat swabs from workers with influenza-like illness (ILI) and NP, throat, and arm swabs from asymptomatic workers for influenza virus by rRT-PCR and sera for seroconversion and antibodies against HPAI A(H5N1) and A(H9N2) viruses.
Results: Among 1273 ILI cases, 34 (2.6%) had A(H5), 56 (4%) had A(H9), and six (0.4%) had both A(H5) and A(H9) detected by rRT-PCR. Of 192 asymptomatic workers, A(H5) was detected in eight (4%) NP and 38 (20%) arm swabs. Of 28 ILI cases with A(H5) or A(H9) detected, none had evidence of seroconversion, but one (3.5%) and 12 (43%) were seropositive for A(H5) and A(H9), respectively.
Conclusion: Detection of AIV RNA in respiratory specimens from symptomatic and asymptomatic LBM workers without evidence of seroconversion or virus isolation suggests environmental contamination, emphasizing caution in interpreting rRT-PCR results in high viral load settings.

Introduction

The first cases of avian influenza A(H5N1) virus infection in humans were detected in 1997, when Hong Kong reported 18 cases linked to live poultry market exposures [1,2]. Two A(H5N1) cases were identified in Hong Kong in early 2003, and since November 2003, more than 870 human cases of A(H5N1) have been reported

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to the World Health Organization (WHO) from 22 countries [3]. Relatively few cases were reported during 2016 through 2021, but 11 cases were reported from eight countries during January 2022 to May 2023, highlighting the ongoing threat posed by A(H5N1) [3].

Despite some cases being mild or asymptomatic, the majority of identified A(H5N1) cases experienced severe illness, with a case fatality proportion of 53% [3]. The primary risk factor for human infection is close, unprotected exposure to infected poultry or virus-contaminated environments, such as live bird markets (LBMs) [4]. A(H5N1) virus is frequently detected in LBMs in Asia, which are a major source of human exposures to the virus [5,6]. Under the International Health Regulations (IHR) 2005, all recent laboratory-confirmed cases of human infection caused by a new influenza A virus subtype, including A(H5N1), must be reported promptly to the WHO [7]. Confirmed cases include those individuals who test positive by polymerase chain reaction (PCR) methods, virus isolation, or paired acute and convalescent serologic tests demonstrating seroconversion [7].

In 2007, the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) in collaboration with the Ministry of Health, Government of Bangladesh, began poultry and environmental surveillance for avian influenza A viruses (AIVs) in LBMs. Subsequently, Bangladesh reported its first outbreak of HPAI A(H5N1) virus in poultry the same year, with A(H5) and A(H9) viruses detected in poultry and market environments year-round since the surveillance began, suggesting ongoing circulation of these viruses among poultry [8].

In 2008, the first human cases of A(H5N1) were detected in Bangladesh, and two more were detected in 2011 [3]. In February 2012, icddr,b initiated surveillance for seasonal and AIVs among 760 workers at 16 LBMs in Dhaka, where surveillance was conducted for AIVs among poultry. During February and March 2012, three LBM workers with influenza-like illness (ILI) had A(H5N1) virus detected by real-time reverse transcription-PCR (rRT-PCR) in their pooled nasopharyngeal and throat swab specimens. Based on the reporting guidance from WHO, these were reported as human cases of A(H5N1). Due to concerns about a possible outbreak among LBM workers, surveillance was expanded, and health authorities distributed oseltamivir to workers with AIVs detected in respiratory swabs by rRT-PCR.

The initial investigation raised concerns that these workers with avian influenza A(H5N1) virus detected in respiratory swabs might not be truly infected, particularly since all of the workers experienced very mild illness, and cycle threshold (Ct) values were high, suggesting very low viral levels. As more workers had AIVs detected at other LBMs by rRT-PCR in respiratory specimens, the Government of Bangladesh consulted with the US Centers for Disease Control and Prevention (CDC) and decided to investigate suspected infections from these markets using serology before reporting to WHO. The Institute of Epidemiology, Disease Control, and Research (IEDCR) and icddr,b jointly launched an investigation at the affected LBMs to (1) describe the human exposures to poultry in these LBMs; (2) expand surveillance for influenza A viruses among LBM workers beyond the surveillance cohort; (3) investigate molecular detections with serologic diagnosis; and (4) describe illness severity and duration of virus detection.

Methods

We conducted two investigations. The first investigation was conducted in LBM A during February-March 2012. The second investigation involved eight markets (LBMs A-H) during November 2012-February 2013. In each market, the investigations continued until 2 weeks after the last detection of AIV in a human respiratory specimen by rRT-PCR.

Qualitative investigation into the context of human exposures to poultry

We conducted a qualitative assessment at LBM A to investigate LBM worker activities in the market and explore potential exposures to AIVs. Following snowball sampling, the field team selected 12 poultry stall workers and owners to interview. In addition, from the line list of ILI cases, the team selected six workers with detectable A(H5) viral RNA in respiratory specimens by rRT-PCR for interviews. The team conducted two group discussions with 18 local poultry stall workers and stall owners who consented to interviews. Through interviews and group discussions, the team assessed LBM workers' daily practices related to exposures to live and dead birds, sources of birds, storing of birds, poultry illnesses and die-offs, and use of personal protective equipment. Additionally, the team conducted 32 hours of observations from six sessions during one week to record the frequency of human-bird interactions, the slaughtering of sick and healthy birds, the use of personal protective equipment, and the market cleaning processes. The qualitative team reviewed the field observation notes, made a list of behaviors and activities related to risk exposures, and then tallied the frequency of the behaviors and activities.

Identifying avian influenza A viruses in poultry

As part of our routine poultry surveillance, we collected poultry and environmental specimens once a month throughout the year. However, during the investigation, we increased the frequency of specimen collections to a weekly basis. Once per week, a field team consisting of a veterinarian and a field assistant approached poultry shop owners to sample poultry. The team collected cloacal swabs from well-appearing, sick, or dead poultry from the LBMs. During these visits, the team also collected environmental swabs from poultry cages, litter, discarded offal, feed, water trays, and fecal materials from poultry stalls in all outbreak markets where an LBM worker had a detectable AIV RNA in nasopharyngeal and throat swab specimens (LBMs A-H).

Poultry and environmental specimens transport and testing

The veterinary team transported the poultry and environmental specimens to the icddr,b animal laboratory, where the specimens were processed and tested separately from the laboratory where human specimens were processed and tested. Viral RNA was extracted from the avian cloacal swab specimens using InviMag Pathogen Kit (Invitek, STRATEC Molecular, Berlin-Buch, Germany) according to the manufacturer's instructions. Initial screening for influenza A virus and subtyping (H5, H7, and H9) of viral RNA was done by one-step rRT-PCR assay using the AgPath-ID™ One-Step RT-PCR kit (Applied Biosystems) (Supplementary Appendix 1 and 2). Testing for subtype A(H9) was not performed for pooled environmental swabs.

Expanding surveillance for influenza-like illness and specimen collection

We expanded ILI surveillance beyond our routine surveillance cohort to screen all workers in the market identified with ILI. Every morning, field staff visited the LBMs, screened all workers in the market for ILI, and collected upper respiratory tract specimens (pooled nasopharyngeal and throat swabs) from ILI cases. We defined an ILI case as an LBM worker with a reported/measured fever and either a cough or sore throat within the past 3 days. LBM workers without a documented fever on the day of screening at the market were still enrolled if they reported a history of fever in

the preceding 3 days. During the second investigation, we broadened the ILI case definition to include LBM workers with any acute respiratory symptoms (e.g., cough, runny nose, sore throat, difficulty breathing, and fast breathing), with or without fever, within 3 days of illness onset. The field staff measured body temperature and recorded clinical symptoms as reported by the LBM workers.

Sampling of healthy control workers

During the second investigation, to determine if having an AIV detected in a respiratory specimen was associated with clinical illness, pooled nasopharyngeal and throat swabs were collected from randomly selected asymptomatic workers matched 1:1 by timing and location within the market to a subset of workers with ILI. We also collected swabs of the upper arms of asymptomatic workers to investigate the potential for body surface contamination with AIVs as an indicator of environmental contamination.

Follow-up of persons with avian influenza A virus detected in swabs

The team followed up LBM workers with ILI detectable influenza A(H5), A(H9), or A(unsubtypeable) viral RNA by rRT-PCR during their acute illness who were available at the market and collected follow-up respiratory specimens (both nasal and throat swabs) at a median interval of every 3 days until the workers became symptom free and/or their swabs tested negative; for workers who had ILI, an acute serum specimen was also collected, followed by a convalescent serum specimen collected 3 or more weeks later.

Human specimen transport and testing

The field team transported all collected respiratory and blood specimens in a cold box at 2-8°C to the icddr,b virology laboratory at the end of each day. At the laboratory, nucleic acids were extracted from respiratory swab specimens using InviMag Pathogen Kit (Invitek, STRATEC Molecular, Berlin-Buch, Germany) on Kingfisher Flex 96 (Thermo Fisher Scientific Inc.) following the manufacturer's instructions. The extracted nucleic acids were used as templates for rRT-PCR assay detection of influenza A viral RNA, targeting M (matrix) genes [9]. Specimens positive for influenza A viral RNA were subtyped by rRT-PCR for A(H1)pdm09, A(H3), A(H5), A(H7), and A(H9) using primers and probes supplied by the US CDC (Supplementary Appendix 1, 2). The PCR master mixtures were prepared with the AgPath-ID™ One-Step RT-PCR kit (Applied Biosystems). rRT-PCRs were performed in CFX96 Touch™ Real-time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The results were analyzed using CFX Maestro Software version 1.1, and the interpretation of the data was performed as per WHO guidelines [9]. Specimens with an appropriate rRT-PCR amplification curve and Ct value ≤38 were considered positive according to the guidelines provided in the "WHO information for the molecular detection of influenza viruses" [10]. Specimens with detectable influenza A viral RNA but unable to be subtyped because of high Ct values were classified as unsubtypeable.

Blood specimens were centrifuged to separate serum, and serum specimens were split into three aliquots and stored at −70°C at icddr,b until one aliquot was shipped frozen on dry ice to the Influenza Division, CDC (Atlanta, GA, USA), for serologic testing. Acute and convalescent sera were tested by hemagglutination inhibition (HAI) and microneutralization (MN) assays against HPAI A(H5N1) clade 2.3.2.1a virus, A/duck/Bangladesh/19097/2013, and low pathogenic avian influenza (LPAI) A(H9N2) virus, A/Bangladesh/0994/2011, in a Biosafety Level III enhanced laboratory. HAI assays were performed following the CDC's modified protocol using horse erythrocytes [11]. MN assays were performed using MDCK cells following protocols as previously described [12]. Serological analysis was conducted with 28 paired sera collected from individuals that were (1) rRT-PCR positive for A(H5) or A(H9) or unsubtypeable and (2) had an acute serum specimen collected within 10 days of symptom onset. A seropositive result for A(H5N1) or A(H9N2) virus was defined as a neutralizing antibody and HAI titer ≥40 (equivalent to WHO protocol criteria of ≥80). Seroconversion against A(H5N1) virus was defined as detection of a ≥4-fold rise in neutralizing antibody titer between acute and convalescent serum, with the convalescent specimen achieving an antibody titer ≥40 [13].

Epidemiologic data analysis

We used chi-squared tests to compare the frequency of symptoms between LBM workers with and without detectable AIVs among all workers with ILI and to compare the proportion of symptomatic and asymptomatic workers with AIV RNA detected in respiratory specimens. We compared the proportion of AIV detection among LBM workers with ILI compared to asymptomatic workers to test the hypothesis that, when detected, AIV infection was the likely etiology of illness rather than the detection of transient environmental contamination of non-viable virus particles. We calculated the correlation coefficient (r) and used the Spearman correlation test to measure the correlation between monthly detections of AIVs in humans, poultry, and market environments. As our data consisted of count values and did not exhibit a linear relationship, we opted for the Spearman correlation coefficient instead of the Pearson correlation [14]. We considered differences with a P-value <0.05 as statistically significant.

Results

Human-poultry interactions from qualitative interviews and observations

LBM A had 66 independent wholesale poultry shops, 37 retail poultry shops, and approximately 1300 workers. The market operated 24 hours a day for 6 days a week and was closed every Monday for cleaning, according to government recommendations. The market sold a variety of birds, including chickens, ducks, swans, and pigeons. An average of 35,280 poultry were transported to LBM A per day, arriving throughout the day and night, from 24 districts across Bangladesh. During the 32 hours of observation, 79 birds (0.2%) were visually sick and 200 (0.5%) were dead on arrival (Table 1). LBM A workers reported that birds arriving sick or dead were also processed and sold. The workers slaughtered poultry by first cutting their throats with a small knife, skinning the carcasses, cleaning the gizzards, and discarding the offal in a plastic drum. The field team observed 56 workers processing 3,009 chickens, and none wore gloves or face masks. There was no source of running water in the market, and hand-washing after handling and slaughtering poultry was rarely observed. Reportedly, at night, 3% (35/1,300) of workers who lived in another district and had nowhere to sleep while in Dhaka slept at LBM A in poultry shops or inside large poultry cages soiled with excreta.

Poultry investigations

Across both investigations, we collected 161 poultry specimens: 58 (36%) had detectable A(H5) viral RNA, nine (6%) had A(H9), eight (5%) had both A(H5) and A(H9), and 15 (9%) had influenza A unsubtypeable viral RNA detected. Of the 98 environmental swabs collected, 34 (35%) tested positive for A(H5), and 18 (18%) had influenza A unsubtypeable viral RNA detected (Supplementary Appendix 3, 4).

Table 1
Types and frequency of reported exposure to poultry among live bird market workers (N = 1300) in Dhaka, Bangladesh, February-March 2012.

	Frequency, n (%)
Poultry characteristics	
Number of poultry present per day	35,280
Number of dead poultry present	200 (0.5)
Number of sick/dying poultry	79 (0.2)
Number of poultry slaughtered	3009 (8.5)
Risk behaviors of live bird market workers, N = 1300	
Number of times touched poultry (all workers)	34,767
Touched lips, nose, eyes, and face after handling poultry	520 (40)
Smoked or shared cigarette between workers	460 (35)
Taking food and drinks in the market	412 (31)
Organizing poultry and cage	150 (11)
Sweeping, swabbing, and cleaning slaughtered area/stall	151 (11)
Touching dead poultry	96 (7)
Put slaughtered chicken in a bag	50 (4)
Weighing poultry	49 (4)
Put slaughtering waste in a bag	39 (3)
Persons slaughtered chicken	35 (3)
Sleeping inside the poultry shop or in/on the poultry cage	27 (2)
Slaughtering sick chicken	17 (1.3)
Cleaned gizzards	17 (1.3)
Frequency of feeding poultry	96 (7)
Use of mask and hand hygiene, N = 1300	
Hand washing without soap	43 (3)
Use of mask	24 (2)
Hand washing after slaughtering poultry	15 (1.1)
Hand washing after feeding poultry	9 (0.7)

Table 2
Proportion of influenza A virus subtypes detected in pooled nasopharyngeal and throat swabs of influenza-like-illness cases in live bird market workers in Dhaka, Bangladesh, February 2012-2013.

Real-time reverse transcription-polymerase chain reaction (rRT-PCR) results	N (%)
Influenza-like-illness episodes	1273
Influenza A viral RNA detected	167 (13)
[a]H1	25 (2)
H3	2 (0.1)
[a]H5	34 (2.6)
H7	0 (0)
[a]H9	56 (4.3)
A/unsubtypeable	67 (5.2)
[a]Co-detections	17 (1.3)

[a] Co-detections: six A(H5) + A(H9) and eleven A(H1) +A(H9)

Detections of avian influenza A virus RNA in human samples

During both investigations, we identified 1273 workers with ILI, with a median age of 28 years (range 21-38 years). Of those 1273, 167 (13%) had influenza A viral RNA detected in their respiratory swabs, with relatively high Ct values (median Ct values: 37, interquartile range [IQR]: 36.1-38). Among the 167 workers with influenza A viral RNA detected, 25 (2%) had seasonal A(H1N1) pdm09, 90 (7%) had AIV RNA [34 (38%) had A(H5), 56 (62%) had A(H9)], and 67 (5%) had influenza A unsubtypeable viral RNAs; 11 with A(H1N1) pdm09 detected also had A(H9) co-detected, and six workers had both A(H5) and A(H9) detected. None had A(H7) detected (Table 2). None of the workers with detectable influenza A viral RNA developed severe illness requiring hospitalization, and the workers continued working during their illnesses. The frequency of ILI symptoms was similar between LBM workers with and without detectable AIV RNA (Table 3).

Detection of AIV viral RNA, including unsubtypeable specimens among LBM workers, coincided in time with the detection of these viruses in poultry (r = 0.81, P = 0.003) and the market environment (r = 0.78, P = 0.007) (Supplementary Appendix 5). Among 192 asymptomatic workers with respiratory swabs collected, patterns of influenza A virus detection were similar to workers with ILI, with both seasonal and AIVs detected with high Ct values. Asymptomatic LBM workers, however, were more likely to have detectable A(H5) viral RNA in respiratory specimens than symptomatic workers (4% vs 2.6%, P = 0.02) (Supplementary Appendix 6). The upper arm specimens from these workers showed a similar pattern of AIV RNA detections as the respiratory swabs, with both seasonal and AIVs detected with high Ct values (Supplemental Appendix 6).

Follow-up swabs from workers with avian influenza A virus detected

For 10 LBM workers with detectable AIV RNA (n = 6) or unsubtypable influenza A viral RNA (n = 4) in respiratory swabs, we collected 76 follow-up respiratory specimens (consisting of one nasopharyngeal and one throat swab) at a median interval of every 3 days (range: 3-18 days). Only one worker had influenza A viral RNA detected in a second swab; both the initial and follow-up swabs were unsubtypeable. Four workers had influenza A viral RNA detected in any follow-up swab specimen, but the detection was usually of a different viral subtype than the first detection. The LBM workers with ILI and detectable influenza viral RNA continued working despite their illnesses, and all follow-up swabs were collected at their workplace (Figure 1).

Serology results

During the first investigation, 28 paired acute and convalescent serum specimens were obtained from workers with ILI who had detectable AIVs in respiratory specimens. The median Ct values for specimens from these cases was 36.5, IQR: 35.2-38. The median duration from symptom onset to first serum collection was 8 days (IQR, 5-9 days) and from first to second serum specimen collection was 31 days (IQR, 26-36 days). None of the cases had evidence of seroconversion against the respective AIVs detected. Nevertheless, 3.5% (1/28) were seropositive for A(H5N1), and 42% (12/28) were seropositive for A(H9N2) at baseline, by both HAI and MN assays (Supplementary Appendix 7, 8).

Discussion

In our investigations, AIV RNA with high Ct values, suggesting low viral levels, was detected in upper respiratory tract specimens collected from symptomatic and asymptomatic workers at LBMs. Arm swab specimens collected from some asymptomatic workers also yielded AIV RNA. In a subset of workers with ILI who provided paired acute and convalescent sera, none had evidence of seroconversion, but a small number had detectable antibodies to some AIVs at baseline. Taken together, these findings suggest that the detection of AIV RNA at low levels in respiratory specimens from asymptomatic and symptomatic LBM workers in Bangladesh may have represented exposure to AIVs and environmental contamination rather than AIV infection.

During 2012, when ILI surveillance detected AIV RNA in respiratory specimens collected from some LBM workers in Bangladesh, there was suspicion that these detections did not represent true human infections, in part because workers were paucisymptomatic. Multiple lines of evidence from subsequent investigations suggested that these detections likely did not represent true infections, including the lack of seroconversion among workers tested and the lack of detection of the same AIVs in follow-up respiratory specimens of individuals who initially tested positive for AIVs with high Ct values. Based on the available evidence from these investigations, no further human A(H5N1) or A(H9N2) cases were reported to the WHO by the Government of Bangladesh after the first three reports.

Table 3
Clinical presentation and outcomes of influenza-like-illness cases (N = 1273) with and without detectable AIV RNA (including unsubtypables) in LBM workers pooled nasopharyngeal and throat swabs in Dhaka, Bangladesh, February-2012-2013.

Clinical presentations/outcomes	LBM workers with detectable AIV RNA, (N = 174), n (%)	LBM workers without detectable AIV RNA, N = 1099 n (%)	*P*-value
Cough	135 (78)	901 (82)	0.16
Runny nose	129 (74)	879 (80)	0.07
Sore throat	45 (26)	362 (33)	0.06
Difficult breathing/shortness of breath	21 (12)	154 (14)	0.48
respiratory rate ≥20	9 (5)	65 (6)	0.69
Conjunctivitis (red eye)	5 (3)	33 (3)	0.92
Hospitalization	0 (0)	0 (0)	-
Death	0 (0)	0 (0)	-

AIV, avian influenza A virus; LBM, live bird market.

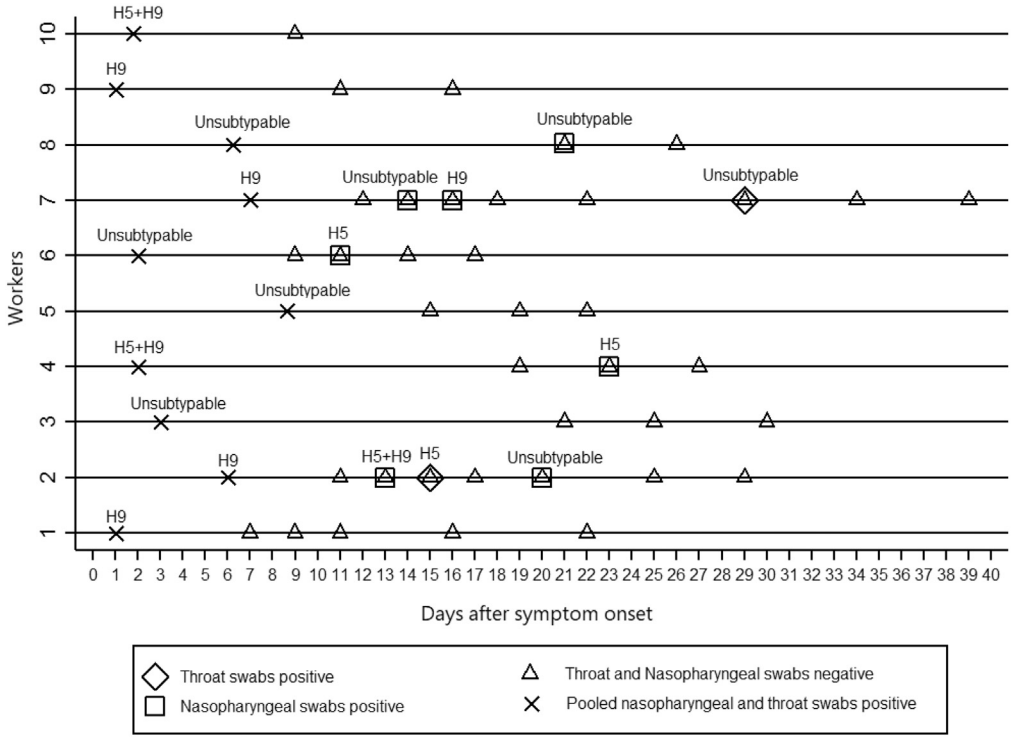

Figure 1. Real-time reverse transcription-polymerase chain reaction (rRT-PCR) detection of avian influenza A virus RNA in serial nasopharyngeal and throat swabs for cases with detectable avian influenza A virus RNA in relation to the onset of illness in live bird market workers in Dhaka, Bangladesh, November 2012-February 2013.
Note: Cycle threshold value ≤38 was considered as a positive result; no A(H7) was detected.

The most likely explanation for the positive rRT-PCR results is that these findings represent the detection of AIV RNA that was deposited transiently in the upper respiratory tracts of workers (asymptomatic and symptomatic) at LBMs that were heavily contaminated with AIVs. The detection of AIV RNA in some workers' upper respiratory specimens coincided with detections of AIV in poultry, and AIV-infected poultry shed viruses in feces that contaminated the environment [15]. Slaughtering of infected poultry can also generate droplets and aerosols with viable viruses, and aerosolized viruses have been isolated from live poultry markets [16–18]. Virus particles containing viral RNA might be deposited on clothing and exposed skin, including arms, through direct contact with infected poultry or by aerosolization during slaughtering and processing of poultry. Virus particles containing viral RNA can also be deposited transiently in the upper respiratory tracts of workers during handling, slaughtering, and de-feathering poultry in the contaminated LBM environment and during handling of poultry without using personal protective equipment. While the RNA is detectable by highly sensitive rRT-PCR assays, the virus particles in which the RNA is contained may be non-viable or do not establish infection. The probable lack of infectivity in the detections described in this study may result from a lack of human cell receptor binding of AIVs, insufficient viral exposure to establish infection, and/or other barriers preventing true infections in the individuals tested.

We detected AIV RNA in respiratory and upper arm specimens collected from asymptomatic workers, providing further evidence of exposure to AIVs in the contaminated environments at LBMs. It was clear from the investigations that LBM workers had ongoing close exposures to poultry, such as handling sick and dead poultry, sleeping inside poultry cages, and slaughtering, defeathering, and eviscerating poultry without using any personal protective equipment. These LBM conditions have not changed substantively since this investigation was conducted, highlighting the continued risk to human health, particularly if a virus that is more transmissible to humans emerges in poultry.

Among the 28 LBM workers with ILI who voluntarily provided paired acute and convalescent sera, only one (3.5%) had evidence

of antibodies to A(H5N1) virus at baseline, and none had evidence of seroconversion. In contrast, 12 of the 28 workers (42%) had positive serology for A(H9N2) virus antibodies at baseline, but there was no evidence of antibody rise in their convalescent sera, indicating no recent infection with A(H9N2) virus. The higher frequency of antibodies to A(H9N2) virus at baseline could be attributable to detection of cross-reactive antibodies to seasonal influenza A viruses or prior A(H9N2) virus infection. Sero-surveys of LBM workers have consistently reported low seroprevalences of antibodies to A(H5N1) virus, ranging from 1.5% in Cameroon and China to 3.7% in Cambodia and 2% in Bangladesh [5,19–22]. A 2020 systematic review and meta-analysis of published serosurveys also reported that the seroprevalences of A(H5N1) virus-specific antibodies were generally low, suggesting that subclinical and clinically mild human A(H5N1) virus infections are uncommon [23].

Our investigations had several limitations. First, we may have missed workers who remained at home due to illness when screening was conducted at the market. Second, we broadened the ILI case definition during the second investigation to include LBM workers with any acute respiratory symptoms (e.g., cough, runny nose, sore throat, difficulty breathing, and fast breathing), with or without fever, within 3 days of illness onset. However, despite the changes to the case definition, we observed similar patterns of illness and AIV detections during both investigations. Third, our attempts to isolate AIVs were unsuccessful, limiting sequencing and further viral characterization, including the specimens that were identified as influenza A positive unsubtypeable. Fourth, the collection of respiratory specimens and arm swabs at the market while LBM workers continued their duties may have limited our ability to avoid any environmental contamination of samples. Implementing a systematic sampling strategy, such as collecting specimens from workers in the morning before entering the market, during working hours, and at the end of the work shift, could provide additional insights into the level of environmental contamination and workers' exposure to AIVs. Finally, our evidence on the duration of viral detection is limited due to the small number of follow-up swabs collected from workers and the presence of different AIV subtypes in subsequent swabs.

Although these investigations were conducted more than 10 years ago, questions about how to interpret the detection of AIV RNA in upper respiratory tract specimens of asymptomatic or mildly ill persons exposed to A(H5N1) virus-infected poultry remain relevant today [24–26]. The 2021-23 widespread outbreaks of HPAI A(H5N1) viruses among wild birds and poultry in previously unaffected regions, coupled with increased spillover to mammals, have raised concerns about the possible appearance of more cases in humans [27–29].

Our results suggest that relying solely on rRT-PCR may overestimate true A(H5N1) virus infections in LBM workers, with or without illness symptoms, particularly for persons exposed to LBM environments that are heavily contaminated with HPAI A(H5N1) viruses. Similarly, recent A(H5N1) virus detection in two asymptomatic poultry farm workers in Spain without serological evidence of infection suggested environmental contamination as a source of exposure [28]. For an accurate diagnosis, the collection of appropriately timed acute and convalescent serum samples for serologic testing by HAI and MN assays at a dedicated public health laboratory may help inform the interpretation of positive rRT-PCR results for A(H5N1) virus. There is, therefore, a need for cautious interpretation of rRT-PCR results in the LBM and other settings where viral load in the environment is high. Factors such as the timing and location of specimen collection, as well as the presence or absence of symptoms, should be taken into consideration. Prospective serial sampling of respiratory specimens collected from both asymptomatic and symptomatic LBM workers and utilizing a combination of virologic and serologic assays and defined criteria for reporting cases would help differentiate AIV infections from environmental contamination and facilitate a more effective public health response. To prevent and control known and potential zoonotic influenza threats and enable an effective public health response, strengthening local surveillance and laboratory capacity to accurately identify human infections with AIVs is needed worldwide.

Declarations of Competing Interest

The authors have no competing interests to declare.

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Ethical approval

The institutional review board of the International Centre for Diarrhoeal Disease Research, Bangladesh reviewed and approved the outbreak investigation protocol, and the Government of Bangladesh reviewed and approved the investigation plan. Study participants provided written informed consent.

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Author contributions

Funding acquisition: SPL, KSR, and ESG. Concept and investigation plan: ESG, KSR, SPL, TMU, and MR. Data curation and analysis: MZH, MAHK, and MSI. First draft manuscript: MZH. Critical revision of the manuscript for important intellectual content: ESG, KSR, SPL, TMU, EAB, ADI, MZL, CTD, MSI, SA, MZR, SC, SUK, MSR, and SN.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the CDC.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2023.08.020.

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