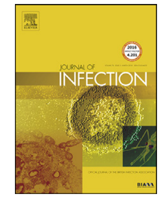
Journal of Infection 79 (2019) 43–48

Contents lists available at ScienceDirect

Journal of Infection

journal homepage: www.elsevier.com/locate/jinf



Transmission risk of avian influenza virus along poultry supply chains in Guangdong, China

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ARTICLE INFO

Article history:
Accepted 10 May 2019
Available online 14 May 2019

Keywords:
Avian influenza virus
Poultry supply chain
Poultry worker
Prevalence
Transmission risk
One health

SUMMARY

Objectives: Avian influenza viruses (AIVs) poise significant risk to human health and the poultry industry. We evaluated the transmission risk along the poultry supply chain.
Methods: During October 2015 and July 2016, four rounds of cross-sectional surveys were performed to characterize AIV spread in farms, transport vehicles, slaughterhouses, wholesale and retail live poultry markets (LPMs). Poultry cloacal and oral swabs, environmental swabs, bioaerosol samples and human sera were collected. Poultry and environmental samples were tested for AIVs by rRT-PCR, further subtyped by next generation sequencing. Previous human H9N2 infections were identified by hemagglutination inhibition and microneutralization tests. Logistic regression was fitted to compare AIV transmission risk in different settings.
Results: AIVs was detected in 23.9% (424/1771) of the poultry and environmental samples. AIV detection rates in farms, transport vehicles, wholesale and retail LPMs were 4.5%, 11.1%, 30.3% and 51.2%, respectively. 5.2%, 8.3% and 12.8% of the poultry workers were seropositive in farms, wholesale and retail LPMs, respectively. The regression analysis showed that virus detection and transmission risk to human increased progressively along the poultry supply chain.
Conclusions: Strengthening control measures at every level along the poultry supply chain, using a one health approach, is crucial to control AIV circulation.

Introduction

Avian influenza viruses (AIVs) are zoonotic pathogens that proliferate in a wide variety of host and undergo continuous genetic reassortment.[1] Currently, 18 different haemagglutination (HA, H1-H18) and 11 different neuraminidase (NA, N1-N11) subtypes have been discovered, among which H1-H16 and N1-N9 were identified in avian species and had adapted to humans, equines, and swine.[2] The continuous reassortment among AIV subtypes resulted in the emergence of novel AIVs and poised significant zoonotic risk to human.[3,4] The H5N1 and H7N9 epidemics have brought substantial damage to the poultry industry and also caused hundreds of human deaths.[5,6] Currently H5, H7 and H9 AIV subtypes were still widely circulating in poultry and various settings along the poultry supply chain,[7,8] and is a major concern for human health and the poultry breeding industry worldwide.

AIVs can contaminate the environment surrounding poultry farm, slaughterhouses, and live poultry markets (LPMs)[9,10] and spread via airborne or contact routes.[11,12] Poultry movement or routines in poultry farms and LPMs, such as wing flapping or feather plucking may generate virus-laden aerosols, while

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https://doi.org/10.1016/j.jinf.2019.05.006
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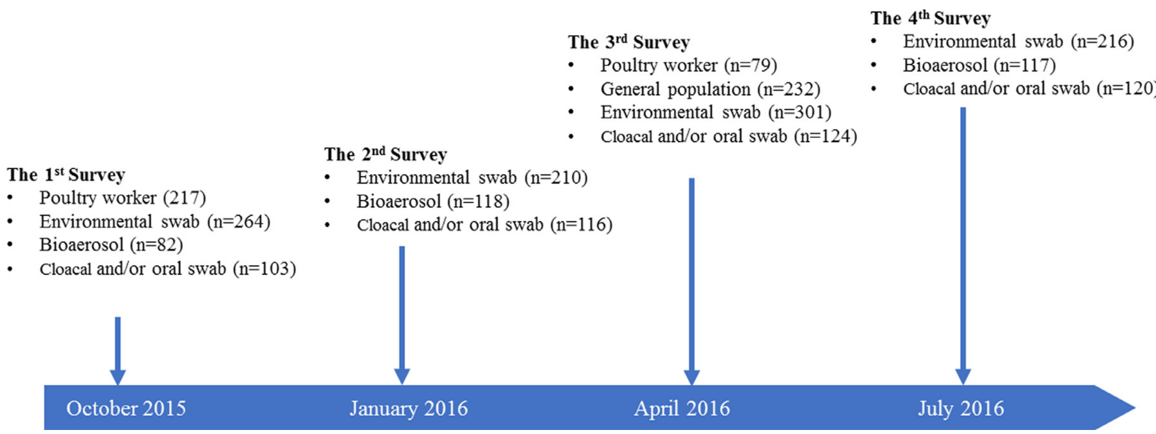

Fig. 1. Timeline of sample collection from poultry, poultry workers, and the environment, 2015–16.

transporting or handling of poultry, disposing poultry droppings, touching contaminated surfaces or contaminated water may also expose poultry workers to AIVs.[13] Poultry workers have long-term occupational exposure to AIVs[14] and hence have high risk to novel avian influenza viruses. Currently, there are still limited studies which characterize AIV contamination in different settings along the poultry supply chain, and factors associated with AIV contamination.[15,16]

Our study characterized AIV detection in poultry farms, transport vehicles, wholesale and retail LPMs, and seroprevalence against influenza A(H9N2), the dominant AIV subtype in poultry,[17,18] among the general population and poultry workers in these settings.

Study design

The study was carried out in October 2015 to July 2016 with 4 rounds of surveys (Fig. 1). We collected samples from 17 poultry farms, 2 wholesale LPMs and 6 retail LPMs in Baiyun, Conghua and Tianhe districts of Guangzhou, China (see Supplementary Appendices 1 and 2 for details). A total of 317 bioaerosol samples (including 82, 118 and 117 in the 1st, 2nd and 4th survey, respectively) were collected using the SKC (SKC, Inc., Eighty Four, PA, USA) BioSampler (SKC catalog number 225-9595) according to the method previously described.[19] The BioSampler was placed at a distance of 1 m from ground and collected a total volume of 240 L of bioaerosols at each sampling occasion (8 L/minute for 30 min). 991 environmental swabs (including 264, 210, 301 and 216 swabs in the 1st, 2nd, 3rd and 4th survey, respectively) from facility surface were collected at each sampling site. We collected the environmental samples by wiping a cotton-tipped polystyrene swab evenly on areas of size approximately 25 cm^2 (5 cm × 5 cm) for 5 times, from different hard surfaces including railings, food troughs, weaning boxes, and gate handles within 1 m of the bioaerosol sampler. 463 cloacal and/or oral swabs (including 103, 116, 124 and 120 swabs in the 1st, 2nd, 3rd and 4th survey, respectively) from poultry were collected from the above 25 poultry facilities. All samples were then transported and shipped within 2 h to the laboratory in a sterile collection tube on ice packs, and then stored under −80 °C for further testing.

Poultry workers from poultry farms, retail and wholesale LPMs in 3 districts (Conghua, Tianhe and Baiyun) of Guangzhou were recruited to the serosurveys. The inclusion criteria were: (1) had been working in poultry farms, retail wholesale LPMs for more than 10 weeks preceding the survey, and exposed to poultry for more than 5 h a week; (2) age ≥16 years (y). We excluded subjects who were immunocompromised, pregnant, suffering from immunosuppressive or immunodeficiency disease (including HIV infection), receiving immunosuppressive therapy, or having acute respiratory tract infection. After receiving informed consent, 3–5 mL blood sample was collected from each participant. During the visits, we also carried out face-to-face interviews by trained personnels, to collect information on demographics, history of exposure to poultry, seasonal influenza vaccination history and other information (see Supplementary Appendix 3 for the questionnaire). Serum samples from 296 individual poultry workers (including 217 and 79 in 1st and 3rd survey, respectively) were collected from farms ($n = 77$), slaughterhouses ($n = 4$), wholesale LPMs ($n = 168$) and retail LPMs ($n = 47$).

We also carried out cross-sectional serosurveys in the general population in the 3rd survey, matched by age and sex of the poultry workers. Subjects from the general population were recruited from those receiving pre-employment medical check in Guangzhou Center for Disease Control and Prevention, under the same exclusion criteria as described above except the criteria on poultry exposure. We collected 232 serum samples and carried out short surveys on demographic information, poultry exposure and influenza vaccination history.

Laboratory analysis

Facilities surface swabs, avian cloacal or oral swabs and bioaerosol were thawed, and total nucleic acid was extracted using the TIANamp RNA Kit for virus detection (Tiangen, Beijing, China). Extracted viral RNA was then assessed with real-time reverse-transcription polymerase chain reaction (rRT-PCR) using influenza virus H5, H7 and H9 subtype nucleic acid detection kit (Jiangsu Shuoshi Biotechnology Co., Ltd, Taizhou, China). AIV-positive samples were further subtyped by using next generation sequencing (NGS) methods of BGI co. Ltd.

Serum samples were subtyped into H5, H7 and H9 using hemagglutination inhibition (HI) assays purchased from Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences. For subtype H9 which was expected to have a higher seroprevalence, samples with HI titer ≥40 were further confirmed by microneutralization (MN) assay, using MN titer ≥40 as the endpoint for seropositivity. Both HI and MN tests were performed according to the WHO Manual for the Laboratory Diagnosis and Virological Surveillance of Influenza.[20]

Statistical analysis

Questionnaire data were managed using EpiData 3.1 software (http://epidata.dk/). Data were entered twice by two independent

Table 1
AIV detection results from poultry and environmental samples.

Variable	*N*	No. positive	Positive %	(95% CI)	OR (95% CI)
All settings and sample types	1771	424	23.9	(22.0–25.9)	
Farms	626	28	4.5	(2.9–6.1)	Reference
Transport vehicles	108	12	11.1	(5.2–17.0)	2.7 (1.3–5.4)
Wholesale LPMs	703	213	30.3	(26.9–33.7)	9.3 (6.2–14.0)
Retail LPMs	334	171	51.2	(45.8–56.6)	22.4 (14.5–34.6)
Poultry cloacal/oral swabs	442	104	23.5	(19.6–27.5)	
Farms	156	10	6.4	(2.6–10.3)	Reference
Wholesale LPMs	190	50	26.9	(20.1–32.6)	5.2 (2.5–10.7)
Retail LPMs	96	44	45.8	(35.9–55.8)	12.4 (5.8–26.3)
Facility surface	991	311	31.4	(28.5–34.3)	
Farms	334	18	5.4	(3.0–7.8)	Reference
Transport vehicles	108	12	11.1	(5.2–17.0)	2.19 (1.0–4.7)
Wholesale LPMs	381	156	40.9	(36.0–45.9)	12.2 (7.3–20.4)
Retail LPMs	168	125	74.4	(67.8–81.0)	51.0 (28.4–91.9)
Bioaerosol[a]	338	9	2.7	(0.9–4.3)	
Farms	136	0	0	–	–
Wholesale LPMs	132	7	5.3	(1.5–9.1)	–
Retail LPMs	70	2	2.9	(0–6.8)	–
Total	1771	424	23.9	(22.0–25.9)	

Abbreviations. OR, odds ratio; CI, confidence interval; LPM, live poultry market.
[a] Odds ratios not calculated due to small sample size.

Table 2
Distribution of AIV positive samples detected by rRT-PCR.

Setting	Subtype, *n*/% (95% CI)								
	N	H5 only	H7 only	H9 only	H5+H7[a]	H5+H9[a]	H7+H9[a]	H5+H7+H9[a]	Untyped
Farms	626	1 0.2 (0.0–0.5)	0	7 1.1 (0.3–1.9)	0	0	0	0	13 2.1 (1.0–3.2)
Transport vehicles	108	0	2 1.9 (0.0–4.4)	2 1.9 (0.0–4.4)	0	0	0	0	8 7.4 (2.5–12.3)
Wholesale LPMs	703	16 2.3 (1.2–3.4)	4 0.6 (0–1.1)	135 19.2 (16.3–22.1)	0	10 1.4 (0.5–2.3)	0	0	48 6.8 (5.0–8.7)
Retail LPMs	334	34 10.2 (6.9–13.4)	11 3.3 (1.4–5.2)	114 34.1 (29.0–39.2)	8 2.4 (0.8–4.0)	25 7.5 (4.7–10.3)	10 3.0 (1.2–4.8)	7 2.1 (0.6–3.6)	41 12.3 (8.8–15.8)
Total	1771	51 2.9 (2.1–3.7)	17 1.0 (0.5–1.4)	258 14.6 (12.9–16.2)	8 0.5 (0.1–0.8)	35 2.0 (1.3–2.6)	10 0.6 (0.2–0.9)	7 0.4 (0.1–0.7)	144 8.1 (6.9–9.4)

Abbreviations. AIV, avian influenza virus; CI, confidence interval; LPM, live poultry market.
[a] Co-detection of multiple AIV subtypes.

persons and verified using structured query language. Questionnaire and laboratory data were later merged into a master dataset, linked by a unique study participant identification number. Data were analyzed using SPSS software version 20.0 (IBM, Chicago, USA). HI and MN titer results were studied as dichotomous outcomes, both using a threshold of ≥1:40 to indicate previous infection.[21] We also selected the most prevalent AIV (H9N2) for further analysis. Univariate logistic regression analyses were performed to identify risk factors associated with previous H9N2 AIV infection confirmed by both HI and MN assays. Multivariable analysis would be carried out if multiple factors were found to be statistically significant in the univariate analysis.

Results

Among all 1771 environmental and poultry samples, 424 (23.9%, 95% Confidence interval (CI) 22.0–25.9%) were detected with AIVs. Among samples collected from poultry (cloacal or oral swabs), 6.4% (95% CI 2.6–10.3%), 26.9% (95% CI 20.1–32.6%), 45.8% (95% CI 35.9–55.8%) were AIV positive in farms, wholesale LPMs and retail LPMs, respectively. Among samples collected from settings with exposure to birds, 4.5% (95% CI 2.9–6.1%), 11.1% (95% CI 5.2–17.0%), 30.3% (95% CI 26.9–33.7%) and 51.2% (95% CI 45.8–56.6%) were AIV positive in farms, transport vehicles, wholesale markets and retail markets respectively. However, AIVs were detected in 9 of 317 bioaerosol samples only, including 7 (5.3%, 95% CI 1.5–9.1%) in wholesale LPMs and 2 (2.9%, 95% CI 0.0–6.8%) in retail LPMs (Table 1).

We detected H5, H7 and H9 AIV from the environmental samples by rRT-PCR (Table 2). Among all poultry and environmental samples, H9 had the highest detection rate (14.6%, 95% CI 12.9–16.2%), followed by H5 (2.9%, 95% CI 2.1–3.7%) and H7 (1.0%, 95% CI 0.5–1.4%). For each subtype, there was a clear increasing trend of AIV detection along the poultry supply chain from farms to retail LPMs. The detection rates increased from 0.2% to 10.2%, 0% to 3.3% and 1.1% to 34.1% for subtype H5, H7 and H9, respectively. Also, co-detections of multiple AIVs were also found, particularly in retail LPMs (Table 2).

The detection rate for H9 was stable over the 4 rounds of surveys (Supplementary Appendix 4). Among the AIV-positive samples, we identified 6 virus subtypes (including 2 H9N2, 2 H7N3, 1 H5N6 and 1 H6N6) by NGS (Supplementary Appendix 5).

In the serosurveys, the participated poultry workers had a median age of 44 years; 69.9% were male. 26.0%, 1.4%, 56.8% and 15.9% poultry workers were recruited from farms, slaughterhouses, wholesale LPMs and retail LPMs, respectively. 15.2% of the subjects had a history of acute respiratory tract diseases in the past 3 months, and 10.8% of subjects had a history of seasonal influenza vaccination, which was higher than the general population (Table 3).

In our serosurvey among poultry workers, only 2 (0.7%, 95% CI 0.0–1.6%) and 1 (0.3%, 95% CI 0.0–1.0%) samples were HI positive for H5 and H7, respectively. 24 (8.1%, 95% CI 5.0–11.2%) of the

Table 3
Characteristics of the poultry workers who have participated in the serological surveys along poultry supply chains (*N* = 296).

Characteristics	*N*	%
Age, y, median (range)	44	58 (15–73)
Gender		
Male	207	69.9
Female	89	30.1
Type of workplace		
Farm	77	26.0
Slaughterhouse	4	1.4
Wholesale LPMs	168	56.8
Retail LPMs	47	15.9
Acute respiratory tract disease in the past 3 months		
Yes	45	15.2
No	251	84.8
Influenza vaccination		
Yes	32	10.8
No	264	89.2

Abbreviations. CI, confidence interval; LPM, live poultry market.

Table 4
Seropositive rate and risk of H9N2 infections among poultry workers along the poultry supply chains[a].

Variable	Seropositive, *n*	% (95% CI)	OR (95% CI)
Sex			
Female	9	10.1 (3.8–16.4)	Reference
Male	15	7.2 (3.7–10.8)	0.7 (0.3–1.7)
Exposure			
General population	3	1.3 (0.0–2.7)	Reference
Poultry worker	24	8.1 (5.0–11.2)	6.3 (1.9–21.1)
Settings			
General population	3	1.3 (0.0–2.7)	Reference
Farms	4	5.1 (0.2–10.2)	4.0 (0.9–18.4)
Wholesale LPMs	14	8.3 (4.2–12.5)	6.4 (1.8–22.8)[b]
Slaughterhouses	0	0.0	–
Retail LPMs	6	12.8 (3.2–22.3)	9.9 (2.4–40.9)
Duration of exposure to poultry (y)			
<10	17	7.9 (4.3–11.6)	Reference
≥10	7	8.5 (2.5–14.6)	1.1 (0.5–2.8)

Abbrevations. OR, odds ratio; CI, confidence interval; LPM, live poultry market.
[a] Exposure and settings were both statistically significant but highly correlated and hence multivariable analysis was not carried out.
[b] Odds ratios not calculated due to small sample size.

poultry workers and 3 (1.3%, 95% CI 0.0–2.8%) subjects from the general populations were tested positive for H9N2 (antibody titers ≥1:40 by both HI and MN tests). 10.1% (95% CI 3.8–16.4%) and 7.2% (95% CI 3.7–10.8%) of the female and male poultry workers, respectively, were H9N2 seropositive. The H9N2 seropositive rates of poultry workers in farms, wholesale LPMs and retail LPMs were 5.1% (95% CI 0.2–10.2%), 8.3% (95% CI 4.2–12.5%) and 12.8% (95% CI 3.2–22.3%) respectively. Seropositive rates were similar irrespective of the duration in the poultry-related occupation (Table 4).

In the regression analysis, environmental samples from transport vehicles (odds ratio [OR], 2.7; 95% CI 1.3–5.4), wholesale markets (OR=9.3; 95% CI, 6.2–14.0), and retail markets (OR=22.4; 95% CI 14.5–34.6) were associated with a higher AIV positive rate than that in farms (all p-values <0.01, Table 1). The increasing ORs along the poultry supply chain suggested that the transmission risk of AIVs progressively increased towards the retail LPMs. The same trends were identified in facility surface and poultry samples (all p-values <0.01, Table 1 and Fig. 2). Comparing to the general population without occupational exposure to poultry, poultry workers had a higher risk of H9N2 seropositivity (OR=6.3, 95% CI 1.9–21.1). Poultry workers in farms had a higher infection risk than the general population (OR=4.0, 95% CI 0.9–18.4) though the results were not statistically significant (p-value = 0.073). Comparing to those working in farms, poultry workers in wholesale and retail LPMs had significantly higher risks of H9N2 infections (OR=6.4; 95% CI 1.8–22.8 and OR=9.9; 95% CI 2.4–40.9 respectively; both p-values <0.005) (Table 4 and Fig. 2).

Discussion

Birds were the main host of AIVs including subtypes H5N1, H5N6, H7N2, H7N9, H9N2, etc. These AIV subtypes have been identified in poultry farms, markets and poultry workers,[9,22,23] but studies with unified observations along the poultry supply chain are limited. In our study, we collected samples at the human–avian–environment interface (poultry workers, LPMs and the environment) along the poultry supply chain, from poultry farms, transport vehicles to wholesale and retail LPMs, to characterize virus amplification and dissemination in these settings.

We found that AIV detection rates increased along with the live poultry supply chain, from about 5% in farms to >50% in retail LPMs. The AIV detection rate from facility surface was higher than those of others, suggesting substantial long-term dissemination and accumulation of AIVs at the endpoint of live poultry trading. Poultry workers in retail LPMs also had a higher seroprevalence of H9 AIV than those in other settings, which suggests that the higher virus detection in the environment did translate to higher infection risk in human with long-term occupational exposure. In particular, the AIV detection rate increased substantially at every setting along the poultry supply chain, indicating that improved cleaning and disinfection at every level is needed to reduce virus amplification.

According to our results, 10.2% of H5 and 3.3% of H7 AIVs were found in retail LPMs. While the prevalence of H5 was similar to those reported in Zhejiang,[13] the prevalence H7 was much lower. However, the reported number of human H7N9 cases was much higher than H5N1 and H5N6 cases in China, suggesting different spill-over risks to poultry workers or the general population. Hence, the interpretation of environmental surveillance data on human infection risk may differ across AIV subtypes. Furthermore, H9 AIV had a much higher detection rate than those of H5 and H7, however human H9N2 infections were usually mild and hence poses a low risk to human. During the study period, poultry were vaccinated against H5N1 only, and a bivalent H5/H7 vaccine was introduced in mid-2017.[18]

In our study, the dominant circulating influenza subtype was H9N2. In June 2016, a laboratory-confirmed H9N2 human case was reported in Guangdong, who had a history of exposure to birds. Exposure history to LPMs could be a major risk factor of H9N2 infection, albeit the virus was less pathogenic to human.[24] However, the circulation of H9N2 remains a risk to the emergence of novel avian influenza as it had contributed internal gene cassettes to H7N9 and H10N8 which led to human death cases. LPM closure and periodic disinfections have been proposed to block AIV transmission to poultry workers and also the general population.[25] For regions where LPM closure was not implemented, cleaning and disinfection after daily closing, and improved sanitation of the LPMs are key measures to control AIV transmission. During the H7N9 epidemic, the Chinese authority implemented the "1110 control measures"[26] – cleaning and sterilizing once a day, cleaning up thoroughly once a week, closing the LPMs once a month, banning overnight holding of live birds to improve the overall market conditions. In addition, other interventions including introduction of 1 or 2 market rest days per month, separating aquatic birds such as live ducks and geese from land-based poultry, promoting chilled chicken to the general population over live chicken have been implemented, which may further reduce the risk of AIV infections in the general population.[15] Our study results show that similar measures should be taken at every level along the poultry supply chain.

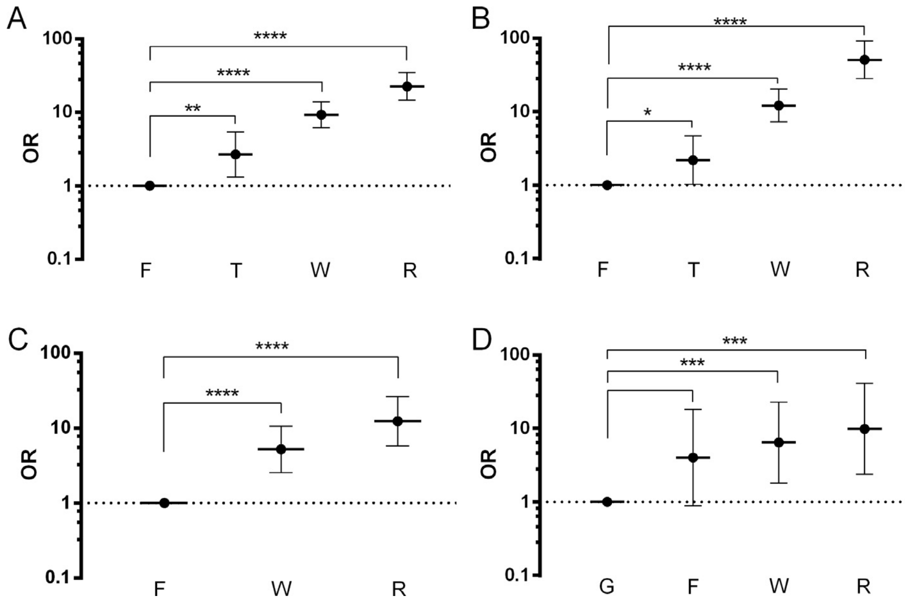

Fig. 2. Risk of AIV detection along poultry supply chain.
A, Settings with poultry exposure; B, Environmental swabs; C: Cloacal or oral swabs from poultry; D: human sera. G: the general population (no occupational exposure to poultry); F: samples collected from farms; T: samples collected from transport vehicles; W: samples collected from wholesale LPMs; R: samples collected from retail LPMs. A, B, C, the reference group was farms; D, the reference group was the general population. The horizontal dotted line indicated OR=1. The results suggested that the transmission risk of AIVs increase along the poultry supply chain, from farms, transport vehicles, the wholesale LPMs to the retail LPMs. * $p<0.05$; ** $p<0.01$; *** $p<0.005$; **** $p<0.001$.

AIV-laden bioaerosol can be suspended in the air for a long time and extend the scope of AIV contamination beyond surfaces. This is particularly difficult to be inactivated especially in the LPM setting where contamination is continuously generated.[27,28] Monitoring AIV aerosol may have the potential as a less invasive method for early detection of AIV in the environment, though optimization of the bioaerosol sampler is needed to increase its sensitivity.

While H5 and H7 AIV subtypes have the ability to cause illness in human,[29–31] no H5 or H7 clinical cases linked to the study sites were reported by Guangdong provincial authority and/or China national authority during our survey period. We found that multiple subtypes of H5, H7 and H9 AIVs co-circulated in the wholesale and retail LPMs, which could increase the risk of reassortment and emergence of novel viruses, especially poultry workers were also exposed to seasonal influenza viruses. Establishing AIV surveillance in retail LPMs would strengthen detection of reassorting events and have the potential to detect rapidly evolved AIVs.[7]

There has been increasing awareness on the interactions between human health, animal health and the environment, and a one health approach should allow better monitoring of emerging zoonotic pathogens.[32,33] The outbreak of H7N9 AIV in China illustrated that the human–animal–environment interface would be the forefront of defense against emerging or zoonotic pathogens.[34] Currently, influenza surveillance in China still mainly relies on hospital-based passive monitoring system, complementary surveillance system at the human–animal–environment interface have the potential to substantially improve detection capacity to emerging zoonotic infections.[33]

This study had some limitations. First, we did not examine the viability of the identified viruses. The detection of viruses in environment cannot translate directly to risk of transmission quantitatively. However, valid comparison for the dissemination of AIV can still be made between different settings along the poultry supply chain. Second, we did not determine the pathogenic characterization of the AIV isolates, so it is uncertain whether the identified viruses were highly pathogenic to humans. Third, duration of bioaerosol sampling may not be long enough to capture sufficient air samples, resulting in lower sensitivity and underestimation of the viral aerosol burden. Fourth, drinking water has been shown to have high prevalence of AIVs,[13] however we have a limited number of samples from each level of the poultry supply chain for meaningful comparison.

Conclusion

Our findings revealed that the transmission risk of AIVs gradually increase along the poultry supply chain from farms, transport vehicles, wholesale LPMs to retail LPMs. H9N2 was the dominant AIV virus subtype among poultry workers, poultry and the environment around poultry facilities. Strengthening biosecurity at every level along the poultry supply chain is a crucial approach to reduce AIV dissemination and accumulation. Long-term surveillance under a One Health approach should be performed to allow monitoring of AIV circulation and identification of modifiable factors for the control of AIV.

Contributors

JY, ZCY and JHL designed and supervised the study. JYW, JY, MLL, CJX, KBL, XWM, JDC, YHL, LC, MXL, BD, YFL, JYL, TGL, XCX and DHQ conducted sample collection, rRT-PCR, HI and MN tests. JYW, EHYL and JY analyzed data and drafted the article, and all authors contributed to review and revision and have approved the final version.

Ethical statements

The ethical approval was approved from the ethics committee of the School of Public Health, Sun Yat-Sen University (2014–18).

Funding

This work was supported by the National Natural Science Foundation of China [grant nos. 81473034 and 31872499], The National Science and Technology Major Project [grant no. 2018ZX10101002], the Guangdong Provincial Science and Technology Project (2018B020241002), the National Key Research and Development Program of China [grant no. 2018YFD0500500], the Science and Technology Program of Guangzhou [grant nos. 201704020056, 201707010451 and 20150010007], the Project for Key Medicine Discipline Construction of Guangzhou Municipality (grant no. 2017-2019-07), Medical Science and Technology Research of Guangdong Province (Grant no. A2016056); and Guangzhou Medical Major Projects (Grant no. 20181A031002).

Conflicts of interest

None declared.

Acknowledgments

We are indebted to the staff of 3 district Centers for Disease Control and Prevention in Guangzhou for collecting blood specimens and related information. We would also like to thank Dr. Shisong Fang from Shenzhen center for Disease Control and prevention for providing H9N2 virus for MN antibody detection.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2019.05.006.

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