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Putative Human and Avian Risk Factors for Avian Influenza Virus Infections in Backyard Poultry in Egypt

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Abstract

Highly pathogenic influenza A virus subtype H5N1 causes significant poultry mortality in the six countries where it is endemic and can also infect humans. Egypt has reported the third highest number of poultry outbreaks (*n*=1,084) globally. The objective of this cross-sectional study was to identify putative risk factors for H5N1 infections in backyard poultry in 16 villages in Damietta, El Gharbia, Fayoum, and Menofia governorates from 2010–2012. Cloacal and tracheal swabs and serum samples from domestic (*n*=1242)and wild birds (*n*=807) were tested for H5N1 via RT-PCR and hemagglutination inhibition, respectively. We measured poultry rearing practices with questionnaires (*n*=306 households) and contact rates among domestic and wild bird species with scan sampling. Domestic birds (chickens, ducks, and geese, $n = 51$) in three governorates tested positive for H5N1 by PCR or serology. A regression model identified a significant correlation between H5N1 in poultry and the practice of disposing of dead poultry and poultry feces in the garbage ($F = 15.7$, $p < 0.0001$). In addition, contact between domestic and wild birds was more frequent in villages where we detected H5N1 in backyard flocks ($F = 29.5$, $p < 0.0001$).

Keywords

Disease reservoirs; Influenza in birds; Influenza A virus – H5N1 subtype; Poultry diseases; Zoonoses

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Conflict of interests

The authors have no conflict of interest to declare.

1. Introduction

Highly pathogenic avian influenza virus of subtype H5N1 (hereafter "H5N1") remains a major public health challenge in many countries. Egypt, which has reported 173 human cases since 2006 with a 36% fatality rate, has reported third highest number of cases in the world after Indonesia and Vietnam as well as the third highest number of poultry outbreaks (*n*=1,084) after Vietnam and Thailand (OIE, 2013; WHO, 2013). Almost all (93%) H5N1 infections in humans in Egypt are associated with poultry exposure (WHO, 2012). The majority of these cases are in women and children, who are the main caretakers of backyard flocks in rural areas (Kandeel et al., 2010; Wilson and Oushy, 2011). This suggests that poultry rearing methods in rural households are important for the persistence of H5N1 in domestic birds and subsequent spillover to humans. In Cambodia, handling and caging backyard poultry were associated with H5N1 infection in humans (Vong et al., 2009). In Thailand, the most significant risk factor for H5N1 in backyard poultry was the occurrence of free-ranging domestic ducks in the flock (Tiensin et al., 2009). In Vietnam, the presence of geese on farms and sharing of scavenging areas with ducks from other farms increased the risk of H5N1 in poultry flocks (Henning et al., 2009). In Indonesia, cropping intensity, elevation, and human population density were significant risk factors for H5N1 in backyard poultry, but unlike Vietnam and Thailand the occurrence of ducks was not a risk factor (Loth et al., 2011). Previous studies of H5N1 in avian hosts in Egypt have analyzed H5N1 prevalence in backyard poultry and wild birds (El-Zoghby et al., 2013), but not their behavioral interactions. To our knowledge, this is the first study of contact between wild birds and poultry. Backyard poultry in rural Egypt typically have direct contact with wild birds because they are allowed to leave the farm household to go outside to feed and swim. In addition to testing domestic birds for H5N1, we sampled wild birds in agricultural fields adjacent to farmers' houses to test for transmission of the virus between wild and domestic birds. Finally, we fielded a questionnaire to attempt to identify possible risk factors for H5N1 at the village level.

2. Materials and methods

2.1. Sampling design

We used a cross-sectional study design, sampling 1,242 poultry and 807 wild birds in 16 villages across four governorates in Lower Egypt from 2010 to 2012 (Table 1). We sampled wild birds and asymptomatic and symptomatic poultry. The governorates sampled were selected based on epidemiological and logistical considerations. To increase the likelihood of detecting H5N1, we selected governorates that had reported a human case of H5N1 in the past year. Of these governorates, we sampled those in which staff from the local branch of the General Organization of Veterinary Services, the Egyptian agency responsible for veterinary health, were available to participate in the planning of field campaigns, liaise with farmers, and take part in field work.

The number of villages sampled per governorate was determined by the governorate's land cover types, defined as ecological regions with characteristic vegetation. We sampled one village in every major land cover type, to test whether land cover affected the prevalence of H5N1 in wild birds. Within a land cover type, villages were selected to be at least 30 km apart by road, but blindly with respect to the presence of H5N1. For example, in Damietta governorate, which has three land cover types - saltwater wetlands bordering the Mediterranean, freshwater wetlands bordering Lake Manzala, and farmland in the Nile Delta - we sampled Ezbet Sita village in a saltwater wetland, Enania village in a freshwater wetland, and Sheta village in farmland.

We sampled houses opportunistically inside each village without inquiring in advance about whether the household flock was infected with H5N1. We sampled all individuals of all wild birds species mist netted at each household. Due to logistic constraints, we could not sample all individuals of all domestic species. Instead, we sampled an average of 4 birds per household and an average of 20 households per village (range: 15–22 households). We administered one questionnaire per household. Each village had up to 1000 backyard birds. A previous study estimated the prevalence of H5N1 in backyard poultry in Egypt at 10.5% (El-Zoghby et al., 2013). Given our sample size and this prevalence, we could be 99% confident of detecting H5N1 if present in backyard flocks (Fosgate, 2009). We took samples (cloacal swabs, tracheal swabs, and serum) from the asymptomatic and symptomatic poultry present in each house. Wild birds were captured with mist nets (12 m × 35mm mesh) open from 5:30–9 a.m. Cloacal and tracheal swabs were placed in viral transport media (VTM), stored in ice in the field, and transported to the National Laboratory for Veterinary Quality Control on Poultry Production where a cold chain was maintained until testing. Serum samples were stored in phosphate buffered saline, centrifuged, and cold chain maintained following established protocols (Brown et al., 2010; Smith et al., 2010).

2.2. Laboratory analysis

Viral RNA was extracted from cloacal and tracheal swabs using a MagNA Pure LC Total Nucleic Acid Extraction Kit with a MagNA Pure LC instrument. All samples were first tested for influenza A presence/absence by a qRT-PCR targeting the M genewith a OneStep Real-Time PCR Kit on a Stratagene MX3005P RT-PCR machine using primers and probes from Spackman et al. (2003). Influenza positive samples were then screened for H5N1 via a second RT-PCR test utilizing the RocheAIV H5N1 RealTime RT-PCR Kit on a Roche LightCycler 2.0 machine. Serological responses to H5-specific antibodies were detected via hemagglutination inhibition (HI) assays using four hemagglutinin units following World Organisation for Animal Health protocols (OIE, 2012). We used a 1% suspension of chicken erythrocytes in V-bottom, 96-wellmicrotiter plates (Hassan et al., 2012). Serum samples were considered positive for H5N1 at a HI titer of ≥ 1:80 (Wang et al., 2012).

2.3. Questionnaires

In each village, we used a 24-question survey to assess study participants' poultry rearing practices, how animals were housed, whether and what kind of protective measures were taken against H5N1 and other diseases, as well as human demographic information such as the number of people per household. At least 15 inhabitants were surveyed at each site (mean = 19, Table 1). The survey also contained questions that the interviewer observed and recorded privately, such as whether poultry were kept in a closed building or fenced-in area. Efforts were made to obtain interviews from the same households in which poultry were sampled, however poultry were observed to be free-ranging and in many cases flocks from different households mixed freely.

2.4. Contact rate analysis

We quantified contact rates among domestic and wild birds inside villages and in surrounding agricultural fields using scan sampling (Martin and Bateson, 2007). We placed 5–10 sampling points between houses in each village and in agricultural fields adjacent to houses. Each point comprised a 50 m diameter circle situated such that the circles' perimeters were at least 100 m apart. The location and behavior of all wild and domestic animals in the sampling plot was recorded, as well as humans.

The research team consisted of one poultry sample collector, two wild bird sample collectors, two researchers who conducted scan sampling, and one researcher who

administered questionnaires. All members of the research team carried out the same roles for all years of the study.

2.5. Statistical analysis

Our goal was to identify putative risk factors for H5N1 in backyard poultry at the village scale. To accomplish this, we fit a regression model to predict the occurrence of H5N1 per village based on predictor variables measured by scan sampling and questionnaires. A village was considered positive for H5N1 if at least one bird from the village was found to be positive by PCR, serology, or both. The regression model was a generalized linear model, which was identical to a logistic regression except that it included a term representing the number of samples per village to account for differences in sampling effort. We measured a total of 289 predictor variables at the village scale. The initial 289 variables were restricted to those that were significant by univariate analysis and uncorrelated. This resulted in the retention of 12 continuous variables (Table 2). Starting from these 12 variables, we carried out stepwise selection to add significant variables to the regression model and remove insignificant ones.

3. Results

3.1 Detection of H5N1 in poultry

The sampled poultry were chickens, domestic ducks, geese, pigeons, and turkeys in backyard flocks. The wild birds were both land birds, principally Passeriformes and Columbiformes, and water birds, Anseriformes and Charadriiformes. We detected H5N1 by RT-PCR in cloacal and tracheal swabs collected from chickens and domestic geese (Fig. 1, Table 1). HA gene sequences were obtained from the positive samples (GenBank accession numbers: JN807778-JN807779). We detected H5N1 by HI tests in serum samples collected from chickens, domestic ducks, and domestic geese. Of these seropositive poultry samples, all had HI titers specific to H5N1 ≥ 80 and 38% had very high titers ≥ 1280 HI units. H5N1 was only detected by RT-PCR in Fayoum, whereas seropositives were more widespread geographically (Fig. 1).

3.2. Variables associated with H5N1 occurrence in villages

H5N1 in backyard flocks was significantly correlated with the practice of disposing of dead poultry or poultry feces in garbage piles outside ($F = 15.7$, $p < 0.0001$) and contact rates between domestic geese and domestic ducks ($F= 29.5$, $p< 0.0001$) (the interaction of these variables was not significant ($F=1.91$, $p=0.17$)). The significant variables were robust to variable selection methods and remained significant when we incorporated differences in sampling effort and detectability of live vs. dead poultry into the regression model. The same variables were important across p_{enter} and p_{leave} ranging from 0.05 to 1.5×10^{-3}. Model specification tests determined that no important variables were omitted from the GLM.

To validate the regression model, we partitioned the surveillance data into a training set comprising 75% of the positive and 75% of the negative villages and tested the model's accuracy on the withheld 25%. The model constructed from the training set had high predictive power when applied to the test set (AUC = 0.852, ROC contrast test chi-square = 176.216, $df = 1$, $p < 0.0001$).

4. Discussion

Since our sampling design was cross-sectional, it is not possible to conclude that there is a causal relationship between H5N1 in backyard poultry in Egypt and the variables that were

significant in our statistical model. However, our results suggest hypotheses that should be tested more specifically in future case-control or cohort studies. The survey respondents routinely disposed of dead poultry or poultry feces in garbage piles either in agricultural fields surrounding the house or the area between neighboring houses. We hypothesize that such outdoor disposal increases the risk of H5N1 spread between households within a village. Backyard poultry in the sampled villages are free-ranging. These birds are released early each morning to forage all day in the area surrounding the family compound. Scan sampling confirmed that free-ranging birds routinely fed on village refuse, where they could potentially contract H5N1 through contact with dead poultry or infected feces in the garbage, particularly as the virus can persist in dead bird feathers for up to 160 days (Yamamoto et al., 2012). A second hypothesis suggested by our results is that contact between domestic geese and domestic ducks leads to H5N1 in backyard poultry because domestic ducks are asymptomatic carriers of H5N1 that transmit the virus to other poultry (Songserm et al., 2006). We routinely observed flocks of domestic ducks and geese swimming together in irrigation canals after being released from family compounds in the morning, providing opportunities for interspecies transmission.

Among the limitations of this study are the short duration of our data collection (June 2010–March 2012) and the fact that sampling was not spaced evenly throughout the year. Although most of our sampling was in the summer, the prevalence of H5N1 in Egyptian poultry is highest during the cold winter months (Hassan et al., 2013). Collecting more samples during this peak time might have allowed us to detect H5N1 in Damietta governorate. Furthermore, our small sample size of 16 villages in four governorates could limit the generalizability of our results. For example, the prevalence of H5N1 in our cloacal swabs was 0.6%, which is similar to the 0.9% prevalence in backyard flocks reported by Kayali et al. (2011), who sampled six governorates, but much lower than that of El-Zoghby et al. (2013), who sampled 24 governorates and found 10.5% prevalence.

In addition, our data set may have been biased toward birds that were easily available to sample. Because active cases of H5N1 are rare we queried the families whose flocks we sampled for currently or recently sick poultry in addition to sampling healthy appearing poultry. We also sampled a single dead chicken found in a garbage pile. This strategy could have resulted in sampling bias if people at some sites failed to report sick poultry, if only some sites had a practice of slaughtering sick poultry, or the practice of slaughtering sick poultry was correlated with disposal method of dead birds. We were able to test for two of these potential sources of bias and found that the practice of killing sick birds was the norm (95%) and the tendency did not vary among sites ($t = 0.1868$, $df = 15$, $p = 0.854$). People who killed sick birds did not dispose of dead birds differently than people who did not (Fisher's exact test: $p = 0.4527$). The dead chicken in which H5N1 was detected was not included in analyses to avoid potential bias.

Our sampling occurred in rural hotspots of H5N1 in humans, which are areas that lack government services such as the removal of refuse to landfills by garbage trucks. Villagers used empty lots as improvised garbage dumps where solid waste including dead poultry and poultry feces from multiple houses was deposited and burned periodically, which is a common garbage disposal practice (Egyptian Environmental Affairs Agency, 2011). Waste collection is inadequate in 73% of villages in rural Egypt and overcrowding increases the amount of solid waste per household (El-Messery et al., 2009). Governorates where we detected H5N1 in backyard flocks in a high percentage of villages had a higher rate of crowdedness, defined as the number of single bedroom households, than those where we detected the virus in few or no villages (El-Gendy, 2011). According to our questionnaires, Fayoum, where we found H5N1 in backyard poultry in 3 villages out of 4 villages, had 11 people per household whereas Damietta, where we found no H5N1, had an average of 5

people. Our model identified the disposal of dead poultry outside in the garbage as a significant risk factor for H5N1 in backyard poultry. An average of 60% of the surveyed households disposed of dead poultry in the garbage (Table 2). We found a significant correlation between the number of people per household and disposal of dead poultry outside in the garbage ($r = 0.38$, $p = 0.00087$). This could be because more crowded households produce more solid waste, including dead poultry and feces. Large solid waste piles that accumulate outside the family compound may attract poultry from neighboring households, which become exposed to birds that died from H5N1, potentially contributing to the virus' persistence in backyard flocks. Disposal of dead birds in the garbage is currently widespread: it is practiced by 42% of households we sampled and 50% of those sampled by Aly et al. (2012) in El Beheira governorate in the Nile Delta. If this hypothesis is confirmed, it would suggest that improving rural waste collection could help stamp out H5N1 in the villages that we sampled. The continuing circulation of H5N1 in backyard birds further stresses the need for sustained control strategies to improve animal and public health throughout Egypt.

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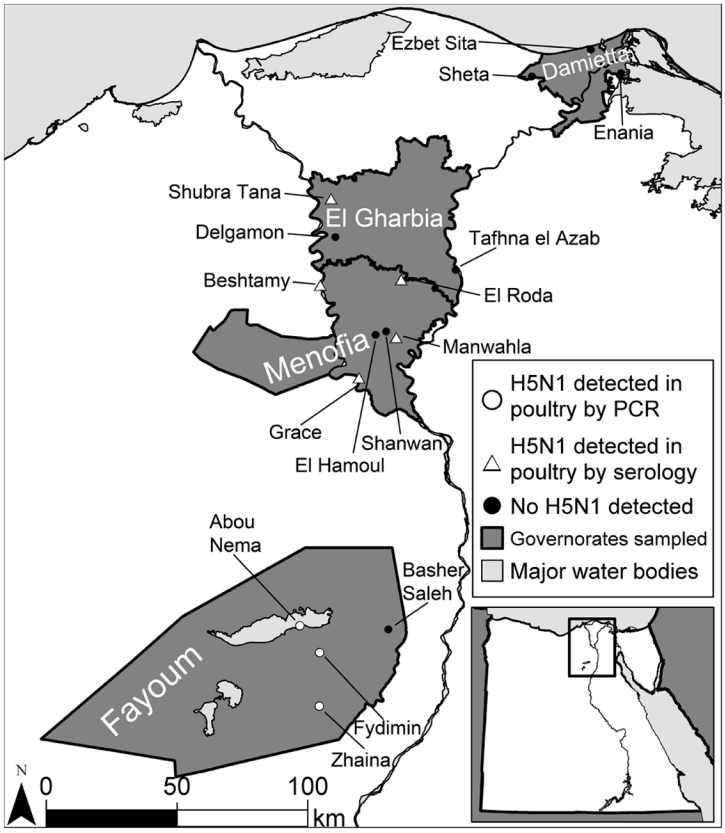

Figure 1.

Main panel: Villages where cloacal swabs, tracheal swabs, and serum samples were collected from wild birds and backyard poultry (2010–2012). Questionnaire surveys and contact rate observations were also conducted at these sites. Inset: location of the sampling sites in Lower Egypt.

Table 1

Number of questionnaires, contact rate observation points, cloacal and tracheal swabs, and serum samples collected at the village scale. H5N1 was detected only in poultry and positives are shown in the last two columns.

Village name	Contact rate observation points	Questionnaires	Wild birds sampled (swabs only)	Poultry sampled (swabs and blood)	H5N1 detected in poultry	
					swabs	blood
Damietta governorate (June 2011)	**60**	**63**	**75**	**102**	**0**	**0**
Ezbet Sita	20	22	30	37	0	0
Enania	20	15	17	25	0	0
Sheta	20	26	28	40	0	0
El Gharbia governorate (July 2011)	**60**	**61**	**109**	**99**	**0**	**13**
Delgamon	20	20	70	31	0	0
Shubra Tana	20	19	0	32	0	13
Tafhna el Azab	20	22	39	36	0	0
Fayoum governorate (April – June 2010)	**60**	**62**	**307**	**491**	**8**	**0**
AbouNema	15	15	91	75	1[1]	0
Basher Saleh	15	15	42	149	0	0
Fydimin	10	22	105	170	7	0
Zhaina	20	10	69	77	1	0
Menofia governorate (February – March 2012)	**114**	**120**	**329**	**550**	**0**	**30**
Beshtamy	20	20	23	119	0	8
El Hamoul	20	19	104	92	0	0
El Roda	21	19	38	77	0	6
Grace	17	22	46	80	0	6
Manwahla	16	20	75	96	0	10
Shanwan	20	20	43	86	0	0
Total	**294**	**306**	**807**	**1242**	**8**	**43**

[1] dead chicken, not included in analyses

Table 2

Starting variables included in the regression model to predict the effect of poultry handling practices and contact between poultry and wild birds on the risk of H5N1 in backyard flocks in Egypt.

	Variable	Mean	Range	Missing data[1] (%)
Questionnaire data	Poultry density (birds/m^2)	0.16	0.03–0.81	0
	Households that dispose of poultry feces outside in the garbage (%)	42	1–67	1.61
	Households that dispose of dead poultry in the garbage (%)	60	15–95	2.66
	Households in which children play with poultry (%)	23	0–52	22.99
	Households that purchase adult birds from the market (%)	1	0–35	4.3
Contact rate data[2]	Households in which domestic ducks had contact with chickens (%)	3.2	0–14	0
	Households in which pigeons had contact with chickens (%)	4	0–18	0
	Households in which wild birds had contact with chickens (%)	5	0–10	0
	Households in which wading birds had contact with chickens (%)	4.6	0–13	0
	Households in which pigeons had contact with domestic ducks (%)	4.2	0–13	0
	Households in which gulls had contact with domestic ducks (%)	0.7	0–2.7	0
	Households in which domestic geese had contact with domestic ducks	2	0–10.3	0

[1] Missing data is the fraction of the households that did not supply this data in our questionnaire. The regression model used the mean of each variable at the village scale, excluding households with missing data.

[2] Frequency of contact was calculated by visually examining a 50m radius scan sampling plot, which was starting by facing north, and proceeding in a clockwise direction, coming full circle over a period of 5–10 minutes depending on environmental complexity and the number of animals in the scan. Each plot was surveyed 5–8 times between 0600 and 1800.