LIVING WITH AVIAN FLU – PERSISTENCE OF THE H5N1 HIGHLY PATHOGENIC AVIAN INFLUENZA
VIRUS IN EGYPT

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Abstract

H5N1 highly pathogenic avian influenza virus (HPAIV) continues to cause mortality in poultry and threaten human health at a panzootic scale in Egypt since it was reported in 2006. While the early focus has been in Asia, recent evidence suggests that Egypt is an emerging epicenter for the disease. Despite control measures, epizootic transmission of the disease continues. Here, we investigate the persistence of HPAIV across wild passerine birds and domestic poultry

between 2009 and 2012 and the potential risk for continuous viral transmission in Egypt. We use a new weighted cross *J*-function to investigate the degree and spatial temporal nature of the clustering between sightings of infected birds of different types, and the risk of infection associated with direct contact with infected birds. While we found no infection in wild birds, outbreaks occurred year round between 2009-2012, with a positive interaction between chickens and ducks. The disease was more present in the years 2010 and 2011 coinciding with the political unrest in the country. Egypt thus continues to experience endemic outbreaks of avian influenza HPAIV in poultry and an increased potential risk of infection to other species including humans. With the current trends, the elimination of the HPAIV infection is highly unlikely without a complete revamp of current policies. The application of spatial statistics techniques to these types of data may help us to understand the characteristics of the disease and may subsequently allow practitioners to explore possible preventive solutions.

Keywords

Influenza A virus, birds, RT-PCR, cross *J*-function, Egypt

Introduction

Outbreaks of the highly pathogenic avian influenza, HPAIV-H5N1 continue in Egypt every year despite various control measures such as culling and vaccination efforts. Since first identified in Asia, HPAIV has caused significant alarm in the health community. While the virus' primary target is birds, it is capable of infecting mammals, including humans, causing serious illness and rates of mortality that may reach alarming proportions. The number of cases of human influenza HPAIV infections is also growing. From 2003 through August 2015, 844 laboratory-confirmed human cases of avian influenza virus infection were officially reported to

the World Health Organization (WHO) from 16 countries, of which 55% (449) have died. Since January 2015, 143 new laboratory-confirmed human cases of avian influenza A (H5N1) virus infection, including 42 fatal cases, have been reported to the WHO. 136 of these new cases are from Egypt and only one is from China. As in previous years, the demographic groups with the highest incidence were young people and women (Dudley 2009; Schroedl 2010; WHO Report 2015). The age range is from one to 75 years, with a median of 26 years, and 23% of the cases are under 10 years of age (WHO Report 2015).

Egypt therefore remains the most affected country HPAIV outside Asia. The country lies at the crossroads of major inter-continental avian flyways linking Africa, Europe, and Asia and as a result represents a likely transmission pathway between regions (Huaiyu et al. 2014). A recent analysis by Cattoli et al. (2011), demonstrated that the virus in Egypt has diverged into two subclades, each of which infect distinct hosts. The “classic” clade 2.2.1.2 circulates in humans and unvaccinated chickens whereas the “variant” clade 2.2.1.1 is present in vaccinated birds that likely appeared due to improper vaccination (Samy et al, 2016). The Nile River Delta where the disease is mostly concentrated has the three risk factors previously identified in Asia as crucial to the proliferation of the virus (Abdelwhab and Hafez, 2011): 1) high densities of domestic waterfowl, 2) high densities of rural human population, and 3) abundance of water and irrigation networks. An analysis of the outbreaks in Egypt reveal similar associations (Abdelwhab and Hafez, 2011), and also highlights elements that are more specific to the Egyptian situation that might be a helpful target for disease prevention and control.

At the human-animal interface, chickens are woven in the fabric of Egyptian society.

Human cases of HPAIV infection often result from contact with infected poultry, particularly in backyard flocks, which are believed to be major reservoirs for the virus, though live bird markets (LBMs) and confined animal feeding operations may also play a role in sustaining the persistence of the virus (ElMasry et al. 2015; Abdelwhab et al. 2010). Currently, HPAIV is not highly transmissible to humans from birds and has a very low rate of human-to-human transmission, (although some cases have been reported). Should a small number of mutations render HPAIV more easily transmissible among humans, there may be a potential for global outbreak. The virus is known to mutate in an intensified manner and is presently considered a potential candidate for a possible pandemic with catastrophic consequences (World Health Organization 2005; Mehle et al. 2012; Linster et al. 2014; Bi et al. 2015).

It is estimated that 4-9.5 million households live with the backyard birds (native chickens, waterfowl and turkeys) all within close proximity with little or no biosecurity measures (Kayali et al. 2011; ElMasry 2015). Furthermore, it has been observed that the Egyptian HPAIV viruses isolated from ducks and humans are closely related to each other, (Ghoneim et al. 2014), suggesting an important epidemiological role of ducks as a reservoir and/or mixing vessel for H5N1 viruses with zoonotic potential.

Most studies have focused on the epidemiology of HPAIV, and more specifically on the factors associated with its presence within Egypt (Yee et al. 2009, Ghoneim et al. 2014). Other approaches, including qualitative and descriptive studies investigating the conditions of HPAIV introduction into Egypt and spread have been used (Abdelwhab et al., 2010, Abdelwhab and Hafez 2011, Cattoli et al. 2011). Empirical studies based on HPAIV distribution data, and more theoretical work focusing on the exploration of disease control scenarios are also being

explored. In addition, the Egyptian government has taken several important steps to confront and control outbreaks in poultry and deal with the occurrence of human cases, but with limited success (Abdelwhab and Hafez 2011). The challenge of controlling HPAIV across such a densely populated region characterized by a diversity of agricultural production systems and economic development remains daunting. Other mechanisms, most probably movement of poultry and poultry products related to human activities are implicated in virus maintenance and distribution in the country.

There is therefore a need for multidisciplinary approaches to explore the ecology of avian influenza viruses and the environment to support ecological interpretation of the source of disease outbreaks in poultry. In this study, we investigate the persistence of AI virus (H5N1) across several bird species between 2009 and 2012 and the potential risk for viral transmission in Egypt. In particular, we investigate the degree and spatial-temporal nature of the clustering between sightings of infected birds of different species

Given the continuous presence of the same strains of HPAIV circulating in Egyptian ecosystems, we hypothesize that regions with little arable land would have higher prevalence of HPAIV due to crowding of backyard flocks in these regions creating conditions conducive to the transmission of HPAI virus.

Methods

Sampling and data collection

Working in collaboration with the Egyptian Animal Health Research Institute/National Laboratory of Quality Control of Poultry Production (AHRI/NLQP), and the General Organization of Veterinary Services (GOVS), we sampled a broad array of common resident and migratory

species as well as domestic poultry in Fayoum, Damietta, El Gharbia, and Menofia governorates between 2009 and 2012 (Fig 1). In selecting sites, we targeted areas of previous HPAIV outbreaks while placing special emphasis on the role of farm size and agricultural practices. The sites were selected after consultation with AHRI/NLQP and GOVS based on their expertise in the HPAIV epizootic. For example, Damietta was selected because its port city is one of the first locations where HPAIV was detected in poultry and was also the site of multiple human cases of HPAIV (Hopp 2010). In each governorate, at least four Districts with multiple towns/villages were sampled. Village research included sampling and testing of wild birds and poultry in village compounds and surrounding farms and natural habitats that represent a gradient of potential interaction between wild and domestic birds. For each site selected, we took geographic coordinates using GPS and: 1) quantified human population densities, using available census information, 2) estimated numbers of poultry and domestic ducks, 3) estimated relative abundance of wild resident and migratory birds, and 4) captured and sampled wild resident and migratory birds (including larger raptors associated with human dominated agricultural areas where possible). Sampling for non-human-associated wild birds included mist-netting along agricultural edges and natural habitats near villages, while domestic birds were sampled from backyards as well as in commercial farms with the assistance of GOVS and AHRI/NLQP staff. In addition to ancillary data (morphometry, blood, feathers) we collected paired cloacal and tracheal swabs per bird in the first year of sampling, but only cloacal swabs in subsequent years, as studies have shown that virus recovery from cloacal swabs is close to as good or just as good as virus recovery from tracheal swabs (Andersen 1996). Birds recaptured were not resampled.

All swabs were placed in cryovials in viral transport medium (Hank's salt solution with antibiotics and fungicides) and chilled on ice within half an hour of collection and transported to the AHRI/NLQP laboratory for analysis within 24 hours of sampling. The swab samples were then tested at AHRI/NLQP using RT-PCR tests for AIV detection, H5 subtype detection and HPAI determination of H5 subtype positives by targeted sequencing.

For the passerines, venous blood was collected in 20-30 µl aliquots using a 50 µl micropipette and sterile tip. The first aliquot of blood was added directly to 450 µl of phosphate buffered saline (PBS). Additional aliquots of blood were sampled and added to the same PBS to achieve a final dilution of 1:10 and mixed briefly by pipetting (Morton et al. 1993; Smith et al. 2010). The diluted blood was later centrifuged at 4°C and the plasma fraction removed for storage at -20°C and later serologic analysis. A volume of PBS equivalent to the plasma fraction was added to the remaining blood cells to maintain a 1:10 dilution and provide a haemostatic buffer.

Molecular testing

Swabs samples were tested by RT-PCR as described elsewhere (Arafa et al. 2010). Briefly RNA was extracted, using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and real-time PCR was performed using Quantitect probe RT-PCR (QIAGEN) according to manufacturer's instructions. Primers and probes used in this study as follows: the primer set for AIV for matrix gene Sep 1 (AGA TGA GTC TTC TAA CCG AGG TCG), Sep2 (TGC AAA AAC ATC TTC AAG TCT CTG) and SePRO (FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA) and PCR condition as follows: 30 min at 50°C, 95°C for 15min, followed by 40 cycles of 95°C for 10sec and 60°C for 30 seconds. The positive samples for matrix gene were further screened for the H5 subtype using the primer set

for AIV (H5) LH1 (ACA TAT GAC TAC CCA CAR TAT TCA G), RH1(AGA CCA GCT AYC ATG ATT GC) and Probe (FAM-TCW ACA GTG GCG AGT TCC CTA GCA- TAMRA)and PCR condition as follows: 30 min at 50°C, 95°C for 15min, followed by 40 cycles of 95°C for 10sec, 54°C for 30 sec and 72°C for 30 seconds.

Real-time RT-PCR was performed using a 7500 Real-time PCR System (Applied Biosystems) and all samples below CT 35 were subjected to further identification.

Samples positive by qRT-PCR were subsequently grown in the allantoic cavities of 10-day-old embryonated chicken eggs in an attempt to isolate influenza A, following established protocols (OIE 2015). Virus isolation was used to confirm the results of RRT-PCR, and to propagate the weak field samples in case of borderline results.

The blood samples collected from poultry and wild birds in 2010-2012 were screened for antibodies against the H5N1 and H9N2 avian influenza subtypes following the protocol established by the Serology Workstream (Smith et al. 2010; Brown et al. 2014). In 2012, serological screening was completed for the six sites in Menofia. Serological data generation was based on avian red blood cell hemagglutination inhibition (HI) assays in accordance with the WHO protocol (OIE 2015), using monoclonal antibodies against influenza virus subtypes H5N1 prepared from the circulating Egyptian virus.

Assessing risk factors related to spread and persistence of AIV in Egypt

At each site we quantified the behavioral and spatial relationships between wild resident and migratory birds, domestic poultry, ducks and humans. Specifically, we quantified the frequency of contact between migratory and resident bird species and domestic animals. Domestic flocks of chickens and muscovy ducks were observed in farm compounds, outside

compounds in the village, and in agricultural settings representing a habitat gradient for potential interactions between wild and domestic birds. All wild birds that came within 20m of each observed domestic flock was identified and the closest distance and duration of proximity recorded. In addition, a large number of variables were collected and analyzed at each site that included farm size, type of animal population, cropping intensity, animal movement between farms, use of animal waste, slaughtering of animals, and other social/cultural/agricultural contact with animals.

Lastly, we also completed 2000 one-hour point counts in 16 core sites within the four Governorates between 2010 and 2012. This approach has seen widespread use in behavioral ecology and indicates the frequency of contact between humans, wildlife and livestock, offering insight into whether there are opportunities for interspecies transmission of H5N1 (Larison et al. 2014). For each of our sites, the count data also provide estimates from 1280 different points in time (i.e. 2 months/per year for 3 years), giving us a spatiotemporally balanced sampling design for exploring both spatial and temporal variation in the bird/human community.

Predictive ecological models to identify at-risk areas for avian influenza in Egypt

To predict areas at risk for avian influenza, we modeled its occurrence using the locations of confirmed avian influenza-positive samples collected in this study. We also analyzed seasonal/yearly shifts in the prevalence of avian influenza. Satellite-based information on precipitation, vegetation characteristics, and standing water was used to identify potential suitable conditions for avian influenza and for different bird groups.

Often these types of data are analyzed using gridded, geostatistical techniques but perhaps additional information can be gleaned from a point process approach which enables

the use of detailed spatial-temporal information on each observation. Viewing each inspected bird as a point in space and time, we apply techniques such as nearest neighbor methods and summary functions which show interactions between infected and non-infected birds and spatial time plots to show how the events change over time. We then used point process methods (Diggle 2013) to describe occurrences of HPAIV in each domestic bird species. In particular, we extended the cross-J function (Van Lieshout 2006), which assumes homogeneity, to the inhomogeneous case, developing a new weighted cross J-function, which can be used to describe the degree of interaction between two different types of birds whose occurrence rates are not constant, as appears to be the case in the data analyzed here. The ordinary cross J -function is one minus the quotient of the nearest neighbor distance distribution function (G -function), and the empty space function (F -function), and is useful for examining clustering or inhibition, relative to the overall rates, of two point patterns N_i and N_j . The ordinary cross J -function assumes that the point patterns are spatially homogeneous. To account for inhomogeneity, we incorporated weights for each point in the point patterns, with each weight corresponding to the inverse of the estimated intensity at its location. For each animal, the intensity estimates were obtained by kernel smoothing the occurrences of detected infections within the species, with a Gaussian kernel and default plug-in bandwidth, using R. To provide a meaningful measure of clustering between point processes N_i and N_j , we propose the following formula for the weighted cross J -function:

$$J_{ij}(r) = 1 - \frac{G_{ij}(r)}{F_j(r)}, \text{ where } G_{ij} \text{ and } F_j \text{ are the weighted cross } G\text{-function and weighted } F\text{-}$$

function, respectively, and thus J_{ij} is estimated using the estimated weighted cross G -function and estimated weighted F -function.

The weighted cross J -function is used to measure the interaction between events in two point processes and to detect clustering or inhibition between them, in order to recognize where spatial interaction appears most prevalent. If $J_{ij}(r) < 0$ then there is clustering between points of type i and type j within distance r . $J_{ij}(r) > 0$ implies that there is inhibition between points of type i and type j within distance r . $J_{ij}(r)$ equals 0 when there is independence between both point processes. We used simulations to demonstrate the successful estimation of the weighted cross J -function. For the clustered simulation, process N_i is simulated from an inhomogeneous Poisson process with a triangular intensity on the square $[0, 105] \times [0, 105]$. N_j is simulated such that for each point τ of N_i , 10 points were simulated independently with a uniform spatial distribution on a circle centered at τ with radius 8.4 km, as shown in Figure 3a. For the inhibition simulation, process N_i is simulated from an inhomogeneous Poisson process with a triangular intensity on the square $[0, 105] \times [0, 105]$. N_i and N_j are simulated by first generating independent Poisson processes with triangular intensity, and then deleting each point of N_j independently with probability 80% if it is within a radius of 8.4 km of any point of N_i (Figure 3b).

Results

In total, we surveyed 346 village sites in 4 governorates (Fayoum, Damietta, El Gharbia, and Menofia governorates) in the Delta region of Lower Egypt (Fig. 1) between 2009 and 2012. Within these villages, we also completed point count surveys in 16 sites spread across the 4 governorates (Fig. 1). During this period, we detected avian influenza virus HPAIV at 59 (14.4%) commercial poultry farms and in 350 (85.6%) backyard flocks (Table 1). The detection percentage by governorate ranged from 10.8% in Damietta to 51.8% in Menofia. Of the positive samples, 45% were from ducks followed by chickens (43.8%), geese (6.8%), turkeys (2.8%) and then pigeons (0.5%). The highest percentage came from backyard flocks (Table 1). In March 2012, we detected the subtype H9N2 virus in Menofia with co-infections (H5N1 and H9N2) across the six sites (Table 2). Interestingly, H9N2 subtype was more dominant than H5N1 in Menofia (mean 62.86% vs 25.01% respectively).

Of the 1350 wild birds (mostly passerines) sampled, none was infected. A data matrix for social and behavioral analysis at the village scale is given in Table 3. We analyzed 432 questionnaires from the four governorates to pinpoint three animal husbandry practices that were significantly associated with H5N1 in backyard flocks (based on the PCR data, published elsewhere): geese density (which was highly correlated with density of other poultry), girls playing with poultry, and disposal of dead poultry in the garbage. All of these practices were highly significant predictors of the occurrence of H5N1 in backyard flocks according to logistic regression (Figure 2).

Serology results

We found no serological evidence of past infection with H5N1 or H9N2 in Fayoum. In Menofia, we detected mean seroprevalence of H5N1 of 25% across six sites and H9N2

seroprevalence of 63% (Table 2). However, there was significant variation among villages with respect to the seroprevalence of both subtypes. Determining the source of this variability in past AIV infection remains an important topic for future research. Because some poultry had been previously vaccinated, we did not carry out any serology on commercial farms. We found no serological evidence of past infection with H5N1 or H9N2 in wild birds

Predictive ecological models to identify at-risk areas for avian influenza in Egypt

Figure 4 shows the infected and non-infected birds within the study area across the four years. Our results show that most of the infection was found in chickens and the least amount of infection was found in wild birds. Within the infected birds, multiple cases of infection were found in chickens and ducks in the southern villages, which might suggest the disease was more dominant in those areas.

To look at how the disease changed over time, we plotted all the infected birds by year. Our results show that the disease was more present in the years 2010 and 2011(Fig 5). We also observed that the disease was more apparent in the southern villages in 2009 and slowly migrates to the northern villages over the following years. This helps us to understand how the disease spread to different regions over time.

As described in our method, a new summary statistic, the weighted cross J -function, was proposed while analyzing this dataset because the point patterns appeared to exhibit non-constant intensity rates. We extended the cross J -function to point patterns where the intensity is not constant, by incorporating weights for each point in the point pattern, with each weight corresponding to the inverse of the estimated intensity at its location, which resulted in more sensible and interpretable results in the case of inhomogeneous point processes. The weighted

cross J -function was applied to each pair of birds in the dataset to inspect for spatial interactions.

As expected, the estimate of weighted cross J -function is less than 0 and shows that most of the clustering occurs before 8.4 km, as shown in Figure 3a. For the inhibition simulation, process N_i is simulated from an inhomogeneous Poisson process with a triangular intensity on the square $[0, 105] \times [0, 105]$. N_i and N_j are simulated by first generating independent Poisson processes with triangular intensity, and then deleting each point of N_j independently with probability 80% if it is within a radius of 8.4 km of any point of N_i (Figure 3b).

Our results detected clustering, that is, a positive interaction between infected chickens and infected ducks within radius of 5.25 km, after inhomogeneity in both the chickens and ducks has been accounted for; there is a lack of apparent interaction at larger distances (Fig. 6a). The results also suggest clustering present between infected geese and infected turkeys within radius of 31.5 km (Fig. 6b). On the other hand, our results suggest repulsion or a negative interaction occurring between infected chickens and infected turkeys (Fig. 6c) and also between infected ducks and infected turkeys (Fig. 6d). Again, the detected interactions are present in the *weighted* estimated cross J -function, meaning that inhomogeneity in the infected animals is not a plausible explanation for the observed interactions. For the interactions between infected ducks and infected geese (Fig. 6e), clustering is detected within radius of 7.5 km, while for infected chickens and infected geese (Fig. 6f), clustering is detected within radius of 8.5 km .

Discussion

The highly pathogenic avian influenza virus HPAIV remains a significant human health issue in Egypt with young people and women being the most affected demographic groups. The disease occurs most frequently in rural areas in the Nile Delta in Lower Egypt where they continue to cause human disease. Our results show backyard poultry (mostly chickens and ducks) play a key role in the persistence of HPAIV in Egypt and agrees with most previous studies (Abdelwhab et al. 2010; Hassan et al. 2012) who also showed that these backyard birds play an important role in further transmission of the virus to other birds in the commercial sector and in live bird markets. The spatial point process model further show that there is clustering between chickens and ducks implying that there may be spread of avian flu between these two groups (Figure 5).

We found no positive infection in wild passerines birds during our sampling efforts, but published data indicates that HPAIV does occur in migratory waterfowl in Egypt. This however, is extremely rare, with just one active HPAIV infection detected after 7,894 waterfowl samples were collected and screened between 2006 and 2011 (Soliman et al. 2012). There is however a possibility that H5N1 may not have been detected in the wild birds because viruses circulating in these species are refractory to growth in chicken eggs. Further studies are warranted to show this. Fayoum, where we first detected active infection in poultry has a high percentage of bare areas such as desert compared to the other sites. Thus, sites surrounded by a high percentage of desert, flocks tend to crowd at high densities into a small amount of land, creating conditions conducive to HPAIV transmission.

In 2012, we detected the subtype H9N2 virus from poultry in Menofia, with co-infections (H5N1 and H9N2). H9N2 in poultry is a potentially important public health issue because

reassortant viruses generated via the exchange of genes between H1N1 and H9N2 have high pathogenicity in mammals (Sun et al. 2011). Indeed, hundreds of migratory birds were recently found dead in China in January 2015 due to exposure to a novel reassortant of HPAIV possessing a Clade 2.3.2.1c HA gene and an H9N2-derived PB2 gene. The novel isolates were also found to be highly pathogenic to both birds and mammals (Bi et al. 2015). Kayali et al. (2014) also identified subtype H9N2 in 2012 in Egypt and suggested that co circulation and co-infection with H5N1 was associated to increased spread and mortality of poultry during September 2012-January 2013. There is already a confirmed human case of H9N2 in south Egypt (WHO 2015), thus further investigation and monitoring is required to prevent this novel reassortant virus from becoming a new threat to public health in Egypt.

Our results also show that HPAIV was more present in the years 2010 and 2011 (Figure 5), and that the disease was more apparent in the southern villages in 2009, slowly migrating to the northern villages over the following years. It is also possible that the political unrest faced in Egypt in 2010 and 2011 may have impacted these results. Indeed, ElMasry et al. (2015) shows that the rate of isolation of HPAIV in the year following the unrest was 4.5 times what it was in previous years. The directional spread of the influenza virus that we detected has also been observed in other regions; for example, there is annual spread of the seasonal influenza virus from northern to southeastern Brazil likely driven by human migration (Alonso et al. 2007).

The continuous detection of HPAIV in this region indicates the possibility that there may be continuous contact with infected poultry, particularly in live bird markets (LBMs) and farms, which are believed to be major reservoirs for the virus (Kandeel et al. 2010; Kayali et al. 2014). Additionally, water has been shown to play a key role in the transmission of low pathogenic

avian influenza viruses (LPAIVs) among waterfowl (Webster et al. 1992, Horimoto and Kawaoka 2001, Rohani et al. 2009; Brown et al. 2014) and has experimentally been implicated in influenza virus transmission among poultry and other bird species. The low pathogenic strains can remain infective in water for extended periods of time but persistence strongly depends on various external factors. There is however, a paucity of information on HPAIV virus presence and stability in water, its route of transmission from bird to humans through water and on associated risks to exposed humans (Leung et al. 2007, Brown et al. 2014).

Another possibility is that HPAIV is endemic in Egypt and the source of the infection is local. For example, fisheries are Damietta's most important industry and with many artificial lakes used for fish farming as well as popular sites for quail hunting. The H5N1 virus can persist in fish and a muddy aquatic environment like that of a fish farm (Horm et al. 2012), which raises the possibility of birds, acquiring the virus while foraging at a fish farm. This hypothesis could be tested by screening fish and water samples from Damietta's fish farms for HPAIV.

A model constructed for Egypt using remote-sensed data on human population density and agricultural production provided accurate predictions of future HPAIV outbreak risk ($R^2=0.69$) (Fuller et al. 2013). This is consistent with previous models of the ecological niche H5N1 in Egypt, which were restricted to the Nile Valley and Delta, but also predicted that areas with intensive agricultural production have a high risk of H5N1 in poultry (Gilbert 2010). The model indicated that unsurveyed areas in the Sinai Peninsula and Red Sea coast (in addition to the well-sampled Nile Valley and Delta) have high ecological suitability for HPAIV and should be priorities for future surveillance.

In this study, a new summary statistic, the weighted cross *J*-function, was proposed and was used to detect apparent clustering between infected chickens and infected ducks, after accounting for spatial inhomogeneity, implying that there may be spread of avian flu between the two groups. Interestingly, ducks have been considered as silent carriers and domestic ducks played a prominent role in regional spread of HPAI virus in Southeast Asia (Gilbert et al. 2006). Indeed, feathers detached from ducks infected with HPAI virus can be a source of environmental contamination, and may function as fomites with high viral loads in the environment, although oro-fecal transmission of avian influenza is likely more important (Yu et al. 2010).

Whenever avian influenza viruses are circulating in poultry, sporadic infections and small clusters of human cases are possible in people exposed to infected poultry or contaminated environments. Although an increased number of animal-to-human infections have been reported over the several years, these influenza A(H5) viruses do not currently appear to transmit easily among people. As such, the risk of community-level spread of these viruses remains low. The increase however in the number of human cases in middle and lower Egypt may likely be attributed to a mixture of factors, including increased circulation of influenza HPAI viruses in poultry, lower public health awareness of risks and seasonal factors such as closer proximity to poultry because of cold weather and possible longer survival of the viruses in the environment. The elimination of the HPAIV infection in poultry in Egypt is unlikely in the unforeseeable future. Therefore, more efforts are required to better understand changes in the evolution and epidemiology. Additional sampling in Upper Egypt would be beneficial because this part of the country has a different culture, climate, and topography from the north, where

our past sampling has occurred. The weighted cross J -function could therefore be used to not only measure the interaction between events in a point process and to detect clustering or inhibition, but also to recognize where spatial interactions occur the most. One important topic for future research is to investigate ways of obtaining approximate standard errors and confidence bands for the weighted cross J -function, in order to determine whether observed clustering or inhibition is statistically significant. Here, our estimates of the weighted cross J -function must be interpreted as purely descriptive. The application of such spatial statistics techniques to these types of data may help us to understand the characteristics of the disease and may subsequently allow practitioners to explore possible preventive solutions.

Our study had several limitations. First, our results indicate we should resample the same sites in Egypt to assess temporal variation in H5N1 and determine whether there are “hotspots” of active H5N1 infections or seroprevalence that persist year after year. Second, future surveillance should sample in Upper Egypt. Lastly, we did not randomly sample our selected sites and this may have included some biases in the results.

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Figures and Tables

Figure 1. Map of scan sampling and questionnaire sites (2010-2012): (A) the four governorates where we collected questionnaires and scan samples, (B) scan and questionnaire sites in Damietta surveyed in 2011 (January-March), (C) sites in Fayoum surveyed in 2010 (April-June), (D) sites in Gharbia surveyed in 2011, and (E) sites in Menofia surveyed in 2012 (February – April).

Figure 2. Poultry handling practices in backyard flocks in Egypt. Variables whose confidence interval does not cross the vertical line at one are significantly associated with H5N1 occurrence in poultry

Figure 3. Estimates of the weighted cross J -function, $J_{ij}(r)$, between simulated N_i and simulated N_j , as a function of radius r , for various choices of pairs of point patterns N_i and N_j . (a) Clustering between simulated N_i and simulated N_j . Red vertical lines corresponds to the clustering threshold of 8.4 km used in the simulations. (b) Inhibition between simulated N_i and simulated N_j .

Figure 4. Maps of Infected and Non-infected birds. The green points represent the locations where infected birds were found. The red points represent the locations where more than one infected bird was found, and the blue points represent the locations where no infected birds were found.

Figure 5. Maps of Infected birds Over Time. Avian Flu (H5N1) was more present in the years 2010 and 2011, and was more apparent in the southern villages in 2009 and appears to migrate to the northern villages in 2010 and 2011.

Figure 6. Estimates of the weighted cross J -function, $J_{ij}(r)$, between N_i and N_j , as a function of radius r , for various choices of pairs of point patterns N_i and N_j . (a) N_i = infected chickens, N_j = infected ducks. (b) N_i = infected geese, N_j = infected turkeys. (c) N_i = infected chickens, N_j = infected turkeys. (d) N_i = infected ducks, N_j = infected turkeys. (e) N_i = infected ducks, N_j = infected geese. (f) N_i = infected chickens, N_j = infected geese.

Table 1. Epizootiologic data for avian influenza virus (H5N1) isolated from Commercial Farms (CF) and Backyard flocks in 4 Governorates in Egypt between 2009 and 2012.

Table 2. Seroprevalence of avian influenza in chickens, geese, and ducks in Menofia in 2012.

Table 3. Data matrix for social and behavioral analysis at the village scale.

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Clustering with Simulated Data

Inhibition with Simulated Data

Infected Geese Over Time

Infected Turkeys Over Time

Infected Chickens Over Time

Infected Ducks Over Time

(a)

Chickens vs Turkeys

Table 1. Epizootiologic data for avian influenza virus (H5N1) isolated from Commercial Farms (CF) and Backyard flocks in 4 Governorates in Egypt between 2009 and 2012.

Variable	Count of H5		Total Count of H5 in Egypt (2009-2012)
	CF Samples (%)	BY Samples (%)	
Governorate	59	350 (85.6)	409
Damietta	3 (0.7)	41 (10)	44 (10.8)
El Gharbia	3 (0.7)	78 (19.1)	81 (19.8)
Fayoum	9 (2.2)	63 (15.4)	72 (17.6)
Menofia	44	168 (41.1)	212 (51.8)
Species	16	163 (39.9)	179 (43.8)
Chickens	35	149 (36.4)	184 (45)
Ducks	4 (0.9)	24 (5.9)	28 (6.8)
Geese	0	12 (2.9)	12 (2.9)
Turkey	0	2 (0.5)	2 (0.5)
Pigeons			
Grand Total	59	350	409

CF= Commercial Farms; BY= Backyards

Table 2. Seroprevalence of avian influenza in chickens, geese, and ducks in Menifia in 2012.

		Site						Mean
		Beshtamy	El Hamoul	El Roda	Grace	Manwahla	Shanwan	
Seroprevalence(%)	H5N1	29.63	0	64.44	21.53	34.44	0	25.01
	H9N2	67.22	48.46	80	100	62.96	18.52	62.86

Table 2. Data matrix for social and behavioral analysis at the village scale.

Data set	Sample size/sites	Spatial resolution	Year
Damietta			
Scan sampling & questionnaire sites	3	Village	2011
Scan sampling points per site (mean)	20		
Questionnaires	63	Village	2011
WHO Human cases (incidence per 10,000 people)	5 (0.04)	Governorate	2007-11
Fayoum			
Scan sampling & questionnaire sites	4	Village	2010
Scan sampling points per site (mean)	15		
Questionnaires	69	Village	2010
Questionnaire sites with data checking completed	69		
Scan sampling & questionnaire sites with positives	3	Village	2010
WHO Human cases (incidence per 10,000 people)	11 (0.038)	Governorate	2006-12
Gharbia			
Scan sampling & questionnaire sites	3	Village	2011
Scan sampling points per site (mean)	20		
Questionnaires	60	Village	2011
Questionnaire sites with data entry completed	3		
Scan sampling & questionnaire sites with positives	0	Village	2011
WHO Human cases (incidence per 10,000 people)	11(0.025)	Governorate	2006-12
Menofia			
Scan sampling & questionnaire sites	6	Village	2012
Scan sampling points per site (mean)	19.5		
Questionnaires	120	Village	2012
Questionnaire sites with data entry completed	4		
Scan sampling & questionnaire sites with positives	6	Village	2012
WHO Human cases (incidence per 10,000 people)	15(0.041)	Governorate	2007-12
Total scan sampling & questionnaire sites	16	Village	2010-12
Scan sampling sites with data entry completed	13		
Questionnaire sites with data entry completed	14		
Total scan & questionnaire site positives	9 positive 7 negative		2010-12
Total WHO human cases	42	Governorate	2006-12