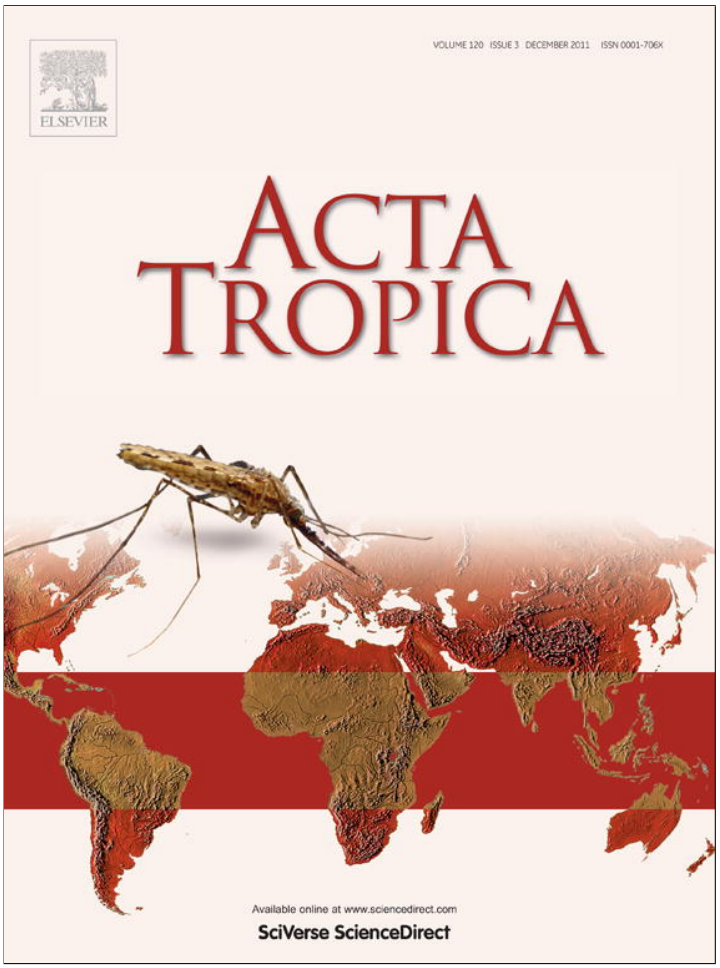

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Looking for avian influenza in remote areas. A case study in Northern Vietnam

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ABSTRACT

Epidemiological surveys of avian influenza infections rarely focus on backyard poultry systems in remote locations because areas with low levels of poultry production are considered to have little influence on the emergence, re-emergence, persistence or spread of avian influenza viruses. In addition, routine disease investigations in remote areas often are neglected due to the lower availability and relatively high cost of veterinary services there. A bank of avian sera collected in 2005 from ethnic minority households in Ha Giang province (Northern Vietnam), located on the Chinese border, was analysed to estimate the seroprevalence of avian influenza virus (AIV) during a H5N1 epidemic and to identify potential risk factors for infection. The results suggest that the chicken population had been exposed to AIV with a seroprevalence rate of 7.2% [1.45; 10.5]. The H5 and H9 subtypes were identified with a seroprevalence of 3.25% [2.39; 4.11] and 1.12% [0.61; 1.63], respectively. The number of inhabitants in a village and the distance to the main national road were the most influential risk factors of AIV infection, and high-risk clusters were located along the road leading to China. These two results suggest a virus spread through commercial poultry exchanges and a possible introduction of AIV from southern China. Remote areas and small-scale farms may play an under-estimated role in the spread and persistence of AIV.

1. Introduction

Based on compulsory notifications of H5N1 highly pathogenic avian influenza (HPAI), avian influenza has been studied widely in Southeast Asia. Multiple sublineages have been isolated from poultry in Vietnam since 2001 (Nguyen et al., 2005, 2008). Despite the mass-vaccination campaigns that have been undertaken since 2005, the virus has never stopped circulating and continues to be detected in non-vaccinated poultry flocks through regular viral monitoring. Vietnam is currently one of the most infected countries in the world, and recorded the second highest number of H5N1 human infections worldwide in 2005 (WHO, 2011). Epidemiological analyses have identified several risk factors of infection whose relative importance changes from one epidemic wave to another (Pfeiffer et al., 2007). These factors, which possibly are favoring the persistence and spread of the disease, include agri-livestock farming systems combining domestic water bird farming and rice production, cold winters, increased poultry stress, and trade before the "Tet" festival period (Gilbert et al., 2006; Henning et al., 2011; Pfeiffer et al., 2007). The Chinese origins of H5N1 viruses isolated in Vietnam has been demonstrated by several groups (Davis et al., 2010; Henning et al., 2011; Nguyen et al., 2005; Phuong, 2005; Wang et al., 2008). However, information on Avian Influenza Virus (AIV) introduction and spread along the Chinese-Vietnamese border is still lacking. This area represents a specific agro-ecosystem, characterized by traditional farming systems and low human and animal population densities. In 2005, the Vietnamese government notified the OIE of 14 outbreaks of H5N1 HPAI in this region: 3 were recorded during the summer of 2005 and 11 during the winter of 2005–2006. Both waves occurred in small village poultry flocks (chickens and ducks) (OIE, 2006a,b).

At the same time as these outbreaks, a sero-bank was established in northern Vietnam under the framework of a French-Vietnamese research project, Biodiva (www.biodiva.org.vn), which aimed to assess and promote genetic diversity in domestic and wild animals. Avian sera in this sero-bank were analysed to assess the epidemiological situation of avian influenza infections in this remote area, and to identify potential risk factors of influenza infection in the low animal density, traditional backyard farming systems found there.

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2. Materials and methods

2.1. Study design

The serological study was performed from April 2005 to August 2006 on poultry bred in northern Vietnam. The study area was Ha Giang province (22°08-23°19′N; 104°33′-105°33′E), which shares a 274 km long border with China (Yunnan province). The topography of the province is characterized by sharp altitudinal variations. According to the government statistics office, the size of the avian domestic population in Ha Giang in 2005 was around 2,140,000, corresponding to a poultry density of 271 head/km^2 (General Statistics Office Of Vietnam, 2005).

Administratively, the province is divided into districts (11), communes (193) and villages (1715). A stratified sampling protocol was applied, with 3 strata: district/commune/village. Within each district, 4 communes were chosen on the basis of official statistics to represent all of the ethnic groups in the province and to cover a wide range of environmental conditions and farming systems. Six to eight villages per commune and 6–8 farmers per village then were randomly selected (Berthouly, 2008). The target sample size was 30–60 chickens in each district. One or two birds were sampled in each sampled household. Interviews designed to collect information on farming practices were conducted on all of the sampled farms by a trained interviewer (Berthouly, 2008).

2.2. Serological assays

The poultry sera were tested against influenza type A with an enzyme-like immunosorbant assay (ELISA) competition test IDVET©. This commercial kit is designed to specifically detect antibodies directed against the NP protein antigen that is highly conserved among all subtypes of influenza type A viruses. Since Vietnamese poultry may be vaccinated with an homologous H5N1 inactivated vaccine, they will produce anti-NP antibodies and will be well-detected by the test (Peyre et al., 2009). The positive serum samples were examined by hemagglutination inhibition (HI) and influenza pseudoparticle-based seroneutralization assay to determine antibody titers and subtypes. The HI test was tailored for H6 and H9 subtypes in poultry by using reference strains presented in the Appendix (Table A.1). The HI test was performed according to standard procedures set down by the World Health Organization (Webster et al., 2002). The detection of H5-specific antibodies was performed by seroneutralization assay with lentiviral-particles pseudotyped with H5 hemagglutinin from H5N1 virus (H5pp) as has been described in detail by Garcia et al. (2010).

2.3. Prevalence estimation in chicken

The real seroprevalence rate for influenza type A was estimated at the individual level using a Bayesian model that accounted for uncertainty about the performance of the ELISA test on exotic strain and host-species. This method requires the definition of beta (a,b) prior distributions for the sensitivity (SE) and specificity (SP) of the test (Branscum et al., 2004). These prior distributions were constructed using BetaBuster software developed by Bayesian Epidemiologic Screening Techniques and available on the website: http://www.epi.ucdavis.edu/diagnostictests/. BetaBuster allows the conversion of confidence intervals obtained from former studies, manufacturer information, and expert opinion into beta prior distribution that then can be used in a Bayesian model. The prior information on SE and SP for avian influenza was obtained from the evaluation the IDVET® test by the national Reference Laboratory for Newcastle Disease and Avian Influenza, Legnaro (Padova), Italy. SE was estimated at 98.70% [$CI_{95\%}$ 92.97; 99.97] and SP was estimated at 98.72% [$CI_{95\%}$ 93.06; 99.97] on chicken sera (Terregino, 2010). The true prevalence model was parameterized and simulated using the "Simulated true prevalence with an imperfect test" calculator, provided by Epitools© software (Sergeant, 2009).

2.4. Epidemiological status characterization

Villages were considered to be distinct epidemiological units. The Standardized Prevalence Ratio (SPR), also known as relative risk, was used to characterize the epidemiological status of each village regarding infection by avian influenza. SPR is defined as the ratio of the observed number of seropositive animals in a village i (Oi) to the expected number of seropositive animals in the same village (Ei) under the assumption of homogeneity of seropositivity prevalence in the region (Bivand et al., 2008).

$$\mathrm{SPR} = \frac{\mathrm{O}i}{\mathrm{E}i}$$

Oi was computed using the number of animals that tested positive in a village i (Pi) adjusted according to SE, SP and the number of animal tested in the village (Ti), to express the true number of seropositive sera (*vs.* apparent) (Dohoo et al., 2003). Ei is the product of the global prevalence in the region by Ti (Bivand et al., 2008).

$$Oi = \frac{Pi + Ti(SP - 1)}{SE + SP - 1}$$

$$Ei = \frac{Ti * \sum_i^n Oi}{\sum_i^n Ti}$$

2.5. Spatial autocorrelation

Longitude and latitude were used to explore the spatial dependence between seropositive cases. The heterogeneity of the relative risk at the village level was assessed first using a Chi-square test (Bivand et al., 2008).

$$\chi^2 = \sum_{i=1}^{n} \frac{(\mathrm{O}i - \mathrm{E}i)^2}{\mathrm{E}i}$$

Particular attention was given to spatially structured heterogeneity: the spatial autocorrelation pattern of SPR was described using Moran's I statistic computed at various spatial lags (i.e. the Moran's test was performed for each kilometer from 0 to 10 km and then for each additional 5 km from 10 to 70 km) (Bivand et al., 2008). Clusters then were identified using Satscan© software (Kulldorff and Information Management Services Inc, 2009).

2.6. Risk factor identification

The relationships between the relative risk of AIV infection at the village level and potential risk factors were assessed using a spatial autoregressive linear model, implemented in R version 2.11.1 (R Development Core Team, 2010). The dependant variable was the log-relative risk of infection (Bivand et al., 2008). Among the available data in the Biodiva database, 9 village-level variables potentially linked to the risk of infection were selected: number of inhabitants, number of households, average family size, main ethnic group, number of different ethnic groups, distance to the main national road, altitude, and average size of chicken and duck flocks. Spatial data, including administrative borders and roads, village census and the number of households within villages were provided by the Vietnamese authorities. Average family size and average size of poultry flocks (chickens and ducks) per household were derived from the household-level data and were

included in the model to characterize the human and animal population (Gilbert et al., 2006, 2008; Nishiguchi et al., 2007). Only partial information related to animal movements and commercial exchanges were available at the village level. Ethnic group therefore was added to the model under the assumption that this variable may represent some specific farming and commercial practices. In addition, the model included a weight term to account for variation in the number of animals tested in each village. Finally, the geographical coordinates and altitude of villages were obtained using a Global Positioning System (GPS).

To determine the most relevant distance range within which spatial autocorrelation should be accounted for in the risk factor analysis, we compared full models including all explanatory variables with various lags for spatial autocorrelation increasing by 2 km from 0 to 40 km. The distance range was chosen according to the values where the Moran's I statistic was significant (p-value < 0.05). Among the 20 tested models, the most relevant one regarding spatial autocorrelation was selected based on the lowest Akaike information criterion (AIC).

Once the optimal range of spatial clustering had been determined, an initial univariate analysis was performed to eliminate non significant potential explanatory variables (p-value < 0.15). The correlation among explanatory variables was checked using the Pearson's product moment correlation coefficient. When covariates were collinear ($r \geq 0.28$), and to eliminate this collinearity, we used the sequential regression method (Graham, 2003). We applied this method in a spatial autoregressive linear models that also included sampling weights. The statistically significant predictors were selected using an automatic stepwise procedure relying on AIC comparisons.

3. Results

A total of 1601 poultry sera collected in 160 villages were analysed. The overall true animal-level seroprevalence of avian influenza infection in the poultry population of the study province was estimated at 7.2% [CI 1.45; 10.5]. The within-village animal-level seroprevalence of AIV ranged from 7% to 98.7%, with an average of 37.3%. The frequency of AIV seroprevalence is shown in Fig. 1 and was mapped in Fig. 2. Among 155 ELISA positive sera, 52 were identified as H5 subtype and 18 as H9 Y280, corresponding, respectively to an overall seroprevalence of at least 3.25% [CI 2.39; 4.11] and 1.12% [CI 0.61; 1.63].

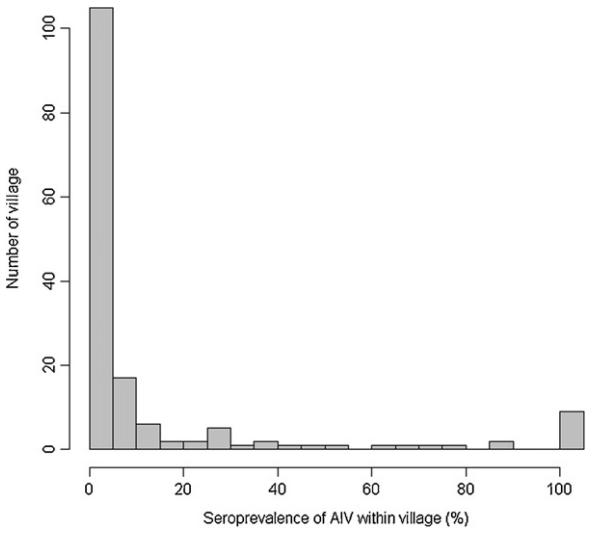

Fig. 1. Frequency of within-village seroprevalence of AIV in 160 sampled villages of Ha Giang province (Northern Vietnam, 2005–2006). The sample size varies from 1 to 26, with a mean of 9 chickens collected in each village.

Table 1
Significant variables (p-value < 0.15) after univariate analyses for the log of the relative risk of AIV.

Covariates	Estimate	p-value
Number of inhabitant in the village	1.9×10^{-3}	<0.001
Distance to the national road	-4.8×10^{-5}	<0.001
Altitude	-5.6×10^{-5}	<0.001
Number of ethnic group	0.35	0.13

The Chi-squared test revealed that the relative risk of AIV infection was heterogeneous across villages (Statistic: 714.8, p-value = 0.001). The spatial correlogramm shows positive significant autocorrelation of SPR up to 8 km (Fig. 3, Appendix (Table A.2)). The coefficients remain positive until 30 km and then the autocorrelation becomes significantly negative for the next 30 km. The model with the lowest AIC (best-fitting model among models with various radius of the spatial autocorrelation term) is obtained with a spatial lag of 5 km (Fig. 4). Therefore the risk factor analysis was undertaken using a spatial autocorrelation term of a 5 km radius. Cluster detection with Satscan© was set up using a radius ranging from 0 to 5 km and identified 10 significant clusters (p-value < 0.05) among which 5 had an increased risk of AIV (SPR > 1) (Fig. 2). High risk clusters are located along the road that leads to the Chinese border, while low risk clusters are in the northeast and southwest of the province.

The main ethnic group living in the village was excluded from univariate analyses. Indeed, at least 9 ethnic groups were represented, making it impossible to compute precise estimates of regression coefficients for each group. As shown in Table 1, univariate analyses identified 4 significant variables (p-value < 0.15): number of inhabitants in the village, distance to the national road, altitude, and number of ethnic groups. The investigation of correlation among potential explanatory variables identified a negative correlation between village population size and the distance to the main national road ($r = -0.41$, p-value < 0.05), meaning that the most densely inhabited villages are located closer to the national road. Significant correlations between each of these two covariates and altitude also were identified ($r = -0.28$, p-value < 0.05, and $r = 0.27$, p-value < 0.05, respectively). According to p-values provided by univariate analyses (Table 1).

Multivariate analyses included at the village level population size, the regression residuals of distance to the national road, and the regression residuals of altitude. The final model obtained after the AIC-based selection procedure included 2 explanatory variables, namely number of inhabitants within the village and the regression residuals of distance to the national road that were statistically significant (p-values < 0.001) (Table 2). For x additional people in a village, the relative risk of within-village AIV seroprevalence would be multiplied by $(1.0025)^x$, the exponential of the statistically significant coefficient related to village size. For an additional 10 people, the relative risk thus increases by 2.5%, and by 25% for an additional 100 people (the average population size of most villages. By the same token, it is estimated that for 1 additional kilometer further away from the national road, the relative risk is reduced by 10% (p-value < 0.001)) and by 100% for 10 additional kilometers.

The spatial correlation term was highly significant in the final model ($\lambda = 0.13$, p-value < 0.001).

4. Discussion

Routine disease investigations and surveillance activities often are neglected in low animal density areas due to the lower

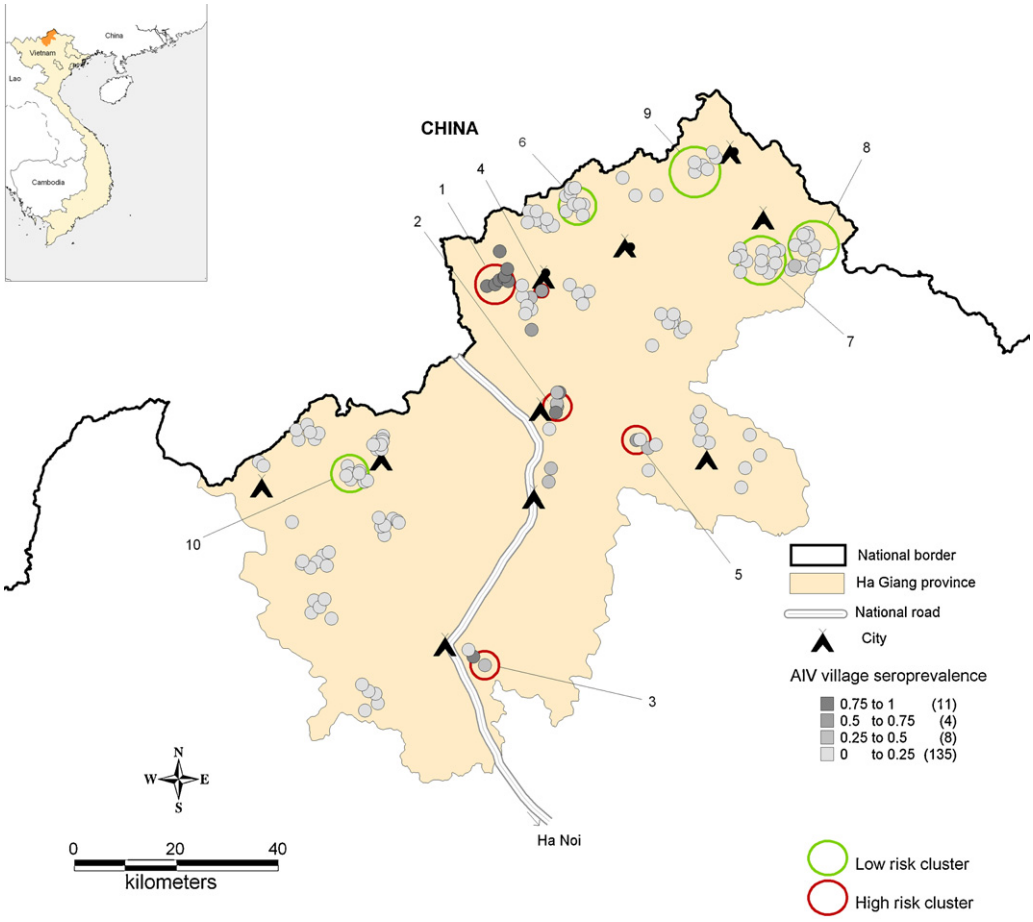

Fig. 2. Seroprevalence of AIV in domestic poultry in Ha Giang province (Northern Vietnam, 2005–2006) and spatial clustering. A relative risk (SPR) >1 indicates an increased risk of being seropositive in comparison with the whole province, while a relative risk <1 indicates a decreased risk.

Cluster	Radius (km)	*P*-value	SPR
1	3.86	0.00	7.25
2	2.79	0.00	5.02
3	2.78	0.00	5.75
4	0.00	0.04	5.02
5	2.76	0.01	2.97
6	3.66	0.00	0.17
7	4.76	0.00	0.07
8	4.88	0.00	0.06
9	4.95	0.01	0.00
10	3.65	0.02	0.00

availability and relatively high cost of veterinary services. In Vietnam, public services are well-staffed all over the country. However, although one member of the veterinary services is present in each commune of Ha Giang province, the number of H5N1 outbreaks in this province may have been underestimated due to a lack of financial and technical resources. We were able to estimate the seroprevalence of AIV infections in such a remote area thanks to the sero-bank provided by the Biodiva project.

At first glance, Ha Giang province may seem to be at a low risk of poultry AIV infection. High animal densities commonly are considered to be a major determinant of AIV persistence and spread (Gilbert et al., 2006; Hogerwerf et al., 2010; Pfeiffer et al., 2007). In our study area, the poultry density is 3-fold lower than the average density at the national level and 20-fold lower than the poultry density in the high-producing area of the Red River Delta (General Statistics Office Of Vietnam, 2005). Furthermore, semi-commercial poultry production systems are almost entirely absent from the

Table 2
Final model for the log of the relative risk of AIV (AIC: 284.48).

Explanatory variable	Estimate	Standard Error	*P*-value	Relative risk for 100 additional units
Number of inhabitants in the village	2.49×10^{-3}	3.82×10^{-4}	<0.001	+24.9%
Regression residuals of the distance to the national road	-3.75×10^{-5}	9.4×10^{-6}	<0.001	−1%

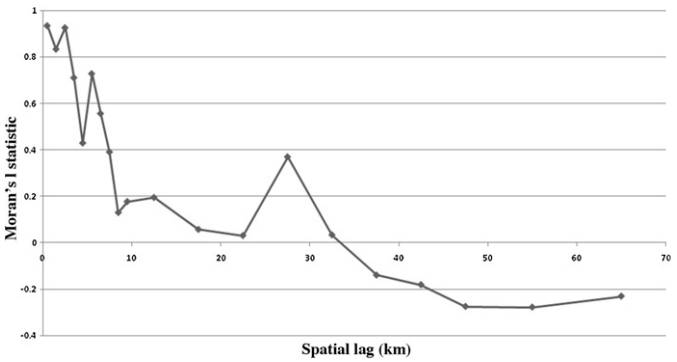

Fig. 3. Spatial correlogram. Series of estimates of Moran' *I* test evaluated for distances ranging from 1 km to 70 km. Moran's *I* is plotted on the vertical axis and the distance on the horizontal axis. A Moran's *I* of zero indicates the null hypothesis of no clustering. A positive spatial autocorrelation, indicates a positive spatial autocorrelation, while a negative coefficient indicates a negative correlation.

province. The mixing of backyard and intensive farming systems is considered to favor virus persistence and spread (Gilbert et al., 2006; Henning et al., 2011; Hogerwerf et al., 2010).

Although Ha Giang province would appear to be a low risk agro-ecological system, our results demonstrate the existence of virus circulation within the poultry population with a seroprevalence rate for AIV estimated at 7.2% [CI 1.45; 10.5]. A similar sero-epidemiological survey conducted in rural households of a mountainous area in Bali (Indonesia), indicates that 15.4% of backyard chickens were seropositive for H5 ($n = 544$) in a context of H5N1 outbreaks (Santhia et al., 2009). The risk of AIV infection in remote areas characterized by low animal densities and traditional family poultry production systems thus may be under-estimated. The role of this type of agro-ecological system in the epidemiology of AIV should not be neglected. Given the epidemiological importance of asymptomatic infections in ducks, it would have been useful to also have estimated the AIV seroprevalence in domestic ducks. Unfortunately, the sera bank provided by the Biodiva project only contained chicken sera.

The seroprevalence rate estimated in the present study should, however, be interpreted cautiously, first because the study was performed in an epidemic context, and second because of the possible imperfect performance of the diagnostic test. The use of a commercial ELISA test can result in a lack of specificity, and thus in an overestimation of the seroprevalence of AIV. Less than half of the ELISA positive sera were confirmed by the HI test (77/155), which identified H5 as the major subtype and the H9 subtype. Moreover, although the first H5N1 vaccination campaign started in 2005, vaccination was implemented first in commercial sector (Peyre et al., 2009) and none of the farmers interviewed had vaccinated their poultry. Furthermore according to the farmer's interviews, replacement chicks are most often produced locally (on the farm-site, or in a farm from same village, or from the same district). Only 0.7% of the replacement chicks are bought on markets (unpublished data), and none comes from China. We thus assumed that they were not vaccinated. The seroprevalence observed for the avian H5 subtype thus most likely reflects the virus spread. The detection of H9 seropositivity is consistent with an infection by H9N2 virus, which is known to circulate in China (Liu et al., 2003), or by H9N3, which already has been isolated in live bird markets in northern Vietnam (Nguyen et al., 2005). The lack of specificity of commercial ELISA kits is a very common drawback that already has been observed in previous studies (Joannis et al., 2008).

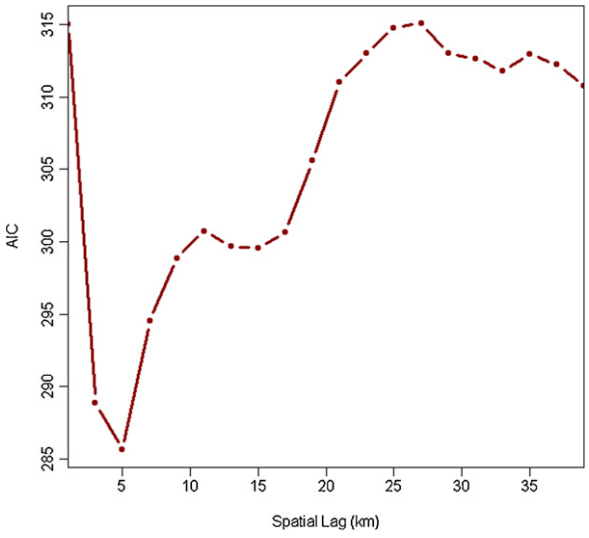

Fig. 4. Akaike information criterion of the complete simultaneous autoregressive model function of spatial lag. The lower AIC is obtained for a spatial autocorrelation of 5th km.

On the other hand, a possible lack of sensitivity of the ELISA test on field samples should be considered. Given that local chickens in Ha Giang have interbred to a great degree with junglefowl (Berthouly et al., 2009), and that the sensitivity of the test varies significantly between bird species, especially in wild birds (Perez-Ramirez et al., 2010; Terregino, 2010), this hypothesis cannot be ruled out.

The seroprevalence estimation also may have been skewed by the survey design. The sample size was calculated for the purposes of genetic analyses, not epidemiological investigations of animal diseases. The minimal within-village seroprevalence observed is 7%. In order to detect at least one infected chicken in a village, the required sample size should have been at least 61 chickens per village (FreeCalc©, (Cameron and Baldock, 1998)). Our sample sizes ranged from 1 to 26, with an average of 9 chickens. Such a low sample size increases the risk of under-estimating village-level seroprevalence. Lastly, due to the difficult terrain and administrative road-blocks, small and isolated villages were excluded from the study. These selection biases may have influenced our findings by over-estimating the animal level seroprevalence.

Regarding AIV determinants, the main risk factors influencing seroprevalence are the number of inhabitants in villages and the distance to the national road. Poultry trade may be the major determinant in the pattern of introduction and spread of H5N1 from south China to Vietnam. This hypothesis already was suggested twice by the molecular tracking of H5N1 virus at the border between the 2 countries (Nguyen et al., 2009; Wang et al., 2008). A social network analysis conducted in North Vietnam, demonstrated the importance of live poultry traders in the virus spread (Soares Magalhaes et al., 2010). Here, we suggest that Ha Giang province may be another port of entry for poultry trade. Populated villages close to the national road may be involved in live animals commercial exchanges, which increase the risk of H5N1infection. In addition, some specific practices in Ha Giang may contribute to virus spread (Berthouly et al., 2009). Further molecular analyses are needed to identify the origin of the strains.

The Chi-square test demonstrated the heterogeneity of the risk of AIV infection and the Moran's *I* test showed that the spatial autocorrelation remains significant 8 km around villages. This suggests a local spread of AIV in villages located 8 km around an infected village. The hypothesis of a local spread of AIV between neighboring villages needs to be considered in the light of the functional links between villages to precise the nature of exchanges. In this area, which is characterized by strong local ethnic identities, steep mountains and unmapped paths, geographical proximity does not

translate automatically into commercial exchanges. The relationships between villages should be investigated further.

5. Conclusion

In Southeast Asia, backyard and family production systems often are in contact with larger commercial and semi-commercial farms, representing an epidemiological context favorable to the occurrence of influenza. This present study provides an opportunity to learn more about avian influenza epidemiology on very small-scale farms in a remote and area with a low level of poultry production. Despite low poultry densities and family farming systems, this specific agro-ecological system can relay avian influenza through small-scale human activities. Our results show both a local and long-distance spread of AIV within the poultry population, possibly due to commercial activities. We conclude that remote areas should not be neglected in animal health surveillance programs. In addition, the study of AIV in traditional farming systems is particularly important in terms of public health because humans and animals are living close together, providing multiple opportunities for direct contact between species.

Acknowledgments

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Appendix A.

Table A.1
Influenza virus strains used for HI test on poultry sera.

Virus name	Subtype	HA Lineage
ATealHK/W312/1997	H6N1	
A/Qa/HK/G1/1997	H9	Gl-hke
A/Dk/HKY280/1997	H9	Y280-like
A Cambodia/408008/2005	H5N1	clade 1

Table A.2
Moran's *I* coefficients and spatial lag.

Spatial lag (km)	Moran's *I* statistic	P-value
0–1	0.9349151	0.002
1–2	0.8338677	0.001
2–3	0.9260044	0.001
3–4	0.7107577	0.001
4–5	0.4292914	0.014
5–6	0.7280945	0.002
6–7	0.5561303	0.001
7–8	0.3907237	0.008
8–9	0.1297051	0.183
9–10	0.1769603	0.084
10–15	0.1945073	0.005
15–20	0.05688737	0.213
20–25	0.03063219	0.378
25–30	0.3696807	0.001
30–35	0.03330221	0.3
35–40	−0.1401482	0.009
40–45	−0.1813129	0.002
45–50	−0.2751489	0.001

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