Vet Res Commun (2008) 32:627–634
DOI 10.1007/s11259-008-9064-8

ORIGINAL ARTICLE

# Environmental and anthropogenic risk factors for highly pathogenic avian influenza subtype H5N1 outbreaks in Romania, 2005–2006

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Accepted: 10 April 2008 / Published online: 5 June 2008


**Abstract** The association between highly pathogenic avian influenza (HPAI) subtype H5N1 outbreak risk in poultry in 161 Romanian villages (October 2005 to June 2006) and environmental and anthropogenic factors was investigated. Village outbreak risk was associated with a village being <5 km from a major road (odds ratio [OR] 5.27, 95% confidence interval [CI] 1.21–22.9) or a river/stream (OR 1.97, 95% CI 1.06–3.72). Outbreak risk in the first part of the epidemic was associated with a village being <5 km from a major road (OR 3.31, 95% CI 1.10–9.98) or a regularly flooded land area (OR 5.08, 95% CI 1.08–23.9); whereas outbreak risk in the second part of the epidemic was associated with a village being <5 km from a river/stream (OR 5.5, 95% CI 1.69–18.9). Results suggest that both environmental and anthropogenic factors influence the risk of HPAI subtype H5N1 outbreaks in village poultry populations.

**Keywords** Influenza · Influenza A virus, H5N1 subtype · Poultry · Disease outbreaks · Risk factors · Romania

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## Introduction

Highly pathogenic avian influenza (HPAI) virus subtype H5N1 is an emerging issue for world health: it has caused numerous disease outbreaks in domestic poultry and wild bird populations, and threatens human health. Between 1997 and early 2008, a total of 365 cases of human infection in 14 countries in South-East Asia, China, central Asia, the Middle East and Africa have been reported to the World Health Organization, resulting in 231 (63%) deaths (World Health Organization 2008). There is a fear that H5N1 could become the next pandemic influenza strain (Alexander 2000). Because exposure to sick or dead poultry is a strong risk factor for human disease caused by HPAI subtype H5N1 (Dinh et al. 2006), the threat of pandemic flu can be reduced by more effectively controlling the spread of HPAI through national poultry flocks. To control the spread of HPAI subtype H5N1 within domestic poultry populations, it is essential to have knowledge of factors that increase the risk of an outbreak occurring. Control programs can then be designed to reduce or avoid exposure to these risk factors, and surveillance programs can be designed to target high risk populations.

Avian influenza virus infection is endemic in a range of free-living bird species world-wide (Alexander 2000), particularly species associated with water − Anseriformes (ducks, geese, swans) and Charadriiformes (gulls, terns, shorebirds)(Stallknecht and Shane 1988). Waterfowl can be infected by all subtypes of type A influenza viruses, with few or no symptoms (Wobeser 1997). These species are capable of spreading influenza viruses between regions (Krauss et al. 2004).

In the northern hemisphere, avian influenza virus infection rates are highest during spring migration for gulls and shorebirds, whereas waterfowl infections peak in late summer and early fall (Krauss et al. 2004). Juvenile waterfowl are more susceptible to infection; when the birds are migrating south, a higher prevalence is observed than in the spring, when the juveniles have matured (Delogu et al. 2003). HPAI outbreaks in poultry are often assumed to occur from contact with wild avian species. The HPAI subtype H5N1 currently circulating in Asia may have arisen from a goose virus in Guangdong, which then infected poultry and reassorted with a H9N2 and/or a H6N1 subtype virus. When the HPAI subtype H5N1 virus infected humans in Hong Kong in 1997, several deaths were reported. The strain was eradicated from poultry, but it continued to circulate in geese. Traditional husbandry practices in China and Southeast Asia, which favour commingling of multiple avian and mammalian species − as well as the popularity of live bird markets − may have contributed to the maintenance of the H5N1 strain (Sims et al. 2005). Suitable habitat is a requirement for the presence of susceptible wild bird species. In addition, the environment must allow contact, either direct or indirect, between potentially infected waterfowl and shorebirds, and domestic poultry species. If domestic species are free-ranging, such contact points might include ponds, lakes, rivers and patches of certain vegetation types.

Outbreaks of transboundary diseases, such as HPAI, provide a unique insight into how animal diseases spread through space and time. Through the information generated, a better understanding of the underlying causes of disease spread can be gained, facilitating the design of control and prevention programs and surveillance systems. The aim of this study was to estimate the association between the risk of outbreaks of highly pathogenic avian influenza subtype H5N1 in poultry in 161 Romanian villages between October 2005 and June 2006 and a range of environmental and anthropogenic factors.

## Materials and methods

The first outbreak of HPAI subtype H5N1 was detected in Romania in early October, 2005. The last outbreak was reported in early June, 2006. Data available for confirmed outbreaks included the outbreak village location (X, Y coordinates), date of occurrence, and species affected. Visual inspection of the epidemic curve revealed 3 epidemic phases: 7 October – 29 December 2005 (days 1 – 84), 30 December 2005 – 16 April 2006 (days 85 – 192), and 17 April – 6 June 2006 (days 193 – 243).

Reported village outbreaks were mapped (ArcGIS™ 9.0. ESRI Inc., Redland CA) using a Romanian county shape file (Dealul Piscului 1970 Datum, Stereographic 70 Projection). Outbreaks were also classified by date of occurrence (days 1 – 192 and 193 – 243) and species affected (chickens only or chickens and other domestic species). Previous research on this epidemic (Ward et al. 2008), using a range of geostatistical visualisation techniques and spatial statistics, indicated that it could be characterised into two parts: disease introduction, local spread and sporadic outbreaks (phases 1 and 2; days 1 − 192), and long-distance disease spread with rapid epidemic propagation (phase 3; days 193 − 243). Therefore, data from epidemic phases 1 and 2 were combined for analysis in the present study. For each outbreak village location, a circular buffer of 10 km radius was created (ArcGIS™ 9.0. ESRI Inc., Redland CA) and up to 4 villages within each buffer were randomly selected (Hawth's Analysis Tools for ArcGIS; http://www.spatialecology.com/htools/). A shapefile of all village locations – case and control – was created. A buffer of 5 km radius was created for all points. This shapefile was overlaid on a series of polyline, polygon and raster coverages in a GIS and relevant information was extracted. These coverages described a range of environmental and anthropogenic variable that might explain the risk of occurrence of a village outbreak of HPAI subtype H5N1. Data were the Romanian road network, which included designated primary and secondary roads, rivers and streams, water bodies (lakes and ponds), elevation and land cover (gData, beta version; http://biogeo.berkeley.edu). The resolution of raster data (elevation, landcover) is 30 seconds. The road network file was processed to exclude roads of unknown primary or secondary status (15% of the database). Road intersections were identified (Hawth's Analysis Tools for ArcGIS; http://www.spatialecology.com/htools/) and a point file was created (ArcGIS™ 9.0. ESRI Inc., Redland CA). All classes represented in the land cover dataset were individually selected and separate raster files were created. These consisted of broadleaf deciduous tree cover, needle leaved evergreen tree cover, mixed leaf type tree cover, herbaceous vegetation cover, regularly flooded shrub and/or herbaceous vegetation cover, cultivated or managed cover, cropland/tree or other cover, and shrub and/or grass cover.

The number of roads, the number of road intersections, and the number of rivers and streams, within 5 km of each case and control location was calculated using a spatial join procedure. The elevation of each case and control location was extracted from the database. All remaining data consisted of raster or polygon files. The area of each variable within 5 km of each case and control location was calculated using a spatial join procedure (ArcGIS™ 9.0. ESRI Inc., Redland CA).

The association between village outbreak occurrence (yes/no) and potential risk factors was estimated by fitting conditional logistic regression models (Egret version 2.0.31. Cytel Software Corporation 1999) to the data and calculating odds ratios (OR) and 95% confidence intervals (CI). A variable identifying the sets of each case village location and up to 4 control village locations within 10 km was created and used as the matching variable. Binary variables for all potential risk factors except elevation and area of cultivated or managed land cover were created, in which '0' represented the absence of the

factor within 5 km of each case or control village location and '1' represented the presence of the variable. For elevation and area of cultivated or managed land cover, the median value of all locations was used to created binary variables in which '0' represented ≤median and '1' represented >median. A multivariate model was fit to the data. Variables associated with the risk of an outbreak at P≤0.20 on bivariate analysis were made available, and a backwards stepwise procedure was used with a P value to enter of 0.10 and P-value to remove of 0.05. The likelihood ratio test was used to select the best fitting model. Standardized model residuals were mapped and a semivariogram was created (Variowin 2.0. University of Lausanne) and visually assessed for evidence of spatial autocorrelation. Moran's autocorrelation statistic (I) for model residuals was also calculated. Conditional logistic regression analysis was repeated for outbreaks occurring during epidemic days 1 − 192 and during epidemic days 193 − 243, and those outbreak in which only chickens were affected and those in which chickens and other domestic poultry species were affected.

The association between the risk of an outbreak affecting >1 poultry species, compared to those affecting only chickens, and the previously described set of factors was estimated by fitting unconditional logistic regression models (Egret version 2.0.31. Cytel Software Corporation 1999) to the data (controls – village outbreaks affecting only chickens; cases – village outbreaks affecting chickens and at least one other species) and the procedure outlined above.

## Results

Of the 161 HPAI subtype H5N1 outbreak villages, control villages within a distance of 10 km were identified for 155. One, 2, 3 and 4 control villages were identified for one (0.6%), 4 (2.6%), 5 (3.2%) and 145 (93.5%) of these 155 outbreak villages, respectively. A total of 48 (31%) and 107 (69%) outbreaks occurred during days 1 − 192 and days 193 − 243, respectively. Most outbreaks (126) included chickens only; in the remaining outbreaks, turkeys (11), ducks (9), guinea fowl (5), geese (2) and turtle-doves (1) were affected.

The following variables were associated (P<0.20) with the risk of an outbreak (Table 1): presence of a road, an intersection, a river or stream, or an area of regularly flooded herbaceous land cover within 5 km. The best fitting multivariate model of outbreak risk included only two variables, presence of a road (OR 5.27, 95% CI, 1.21 − 22.9; P=0.0266) and presence of a river or stream (OR 1.97, 95% CI, 1.05 − 3.72; P=0.0382) within 5 km. An interaction term between these two variables was not significantly (P=0.292) associated with outbreak risk. No significant (P=0.8139) spatial autocorrelation (I=0.004) was detected for the model standardized residuals.

For outbreaks (n=48) occurring between epidemic day 1 and 192, the following variables were associated (P<0.20) with the risk of an outbreak: presence of a road, an area of broadleaf tree cover, an area of mixed leaf tree cover, an area of flooded shrub or herbaceous land cover, and an area of cropland, tree or other natural vegetation land cover within 5 km. The best fitting multivariate model of outbreak risk included only two variables, presence of a road (OR 3.31, 95% CI, 1.10 − 9.98; P=0.0333) and presence of an area of flooded shrub or herbaceous land cover (OR 5.08, 95% CI, 1.08 − 23.9; P=0.0397) within 5 km. An interaction term between these two variables was not significantly (P=0.399) associated with outbreak risk. No significant (P=0.5741) spatial autocorrelation (I=0.02) was detected for the model standardized residuals. For outbreaks (n=107) occurring between epidemic day 193 and 243, the following variables were associated (P<0.20) with the risk of an outbreak: presence of a road, a road intersection, a river or stream, or an area of mixed leaf tree cover within 5 km. The best fitting multivariate model of outbreak risk included only the presence

**Table 1** Association between road, water feature, land cover and elevation variables and the risk of an outbreak of highly-pathogenic avian influenza subtype H5N1 in any domestic avian species in Romanian villages, 2005 − 2006

| Variable | Level | Outbreak villages | Control villages | Odds ratio | P-value |
|---|---|---|---|---|---|
| No. roads | >1 | 152 | 556 | 4.37 | 0.0643 |
| | 0 | 3 | 48 | … | … |
| No. road intersections | >1 | 79 | 76 | 1.34 | 0.0093 |
| | 0 | 264 | 340 | … | … |
| No. lakes and ponds | >1 | 29 | 126 | 0.99 | 0.5208 |
| | 0 | 114 | 490 | … | … |
| No. rivers and streams | >1 | 133 | 22 | 1.69 | 0.0052 |
| | 0 | 472 | 132 | … | … |
| No. areas of broadleaf tree cover | >1 | 108 | 47 | 1.10 | 0.2433 |
| | 0 | 408 | 196 | … | … |
| No. areas of evergreen tree cover | >1 | 41 | 114 | 0.99 | 0.9395 |
| | 0 | 161 | 443 | … | … |
| No. areas of mixed leaf tree cover | >1 | 56 | 99 | 0.91 | 0.6154 |
| | 0 | 232 | 372 | … | … |
| No. areas of herbaceous land cover | >1 | 12 | 143 | 1.32 | 0.3007 |
| | 0 | 36 | 568 | … | … |
| No. areas of regularly flooded land cover | >1 | 8 | 147 | 1.88 | 0.0581 |
| | 0 | 17 | 587 | … | … |
| No. areas of cultivated land cover* | >122 | 74 | 81 | 0.92 | 0.4234 |
| | ≤122 | 301 | 303 | … | … |
| No. areas of cropland/tree/other land cover | >1 | 12 | 143 | 1.45 | 0.2348 |
| | 0 | 33 | 571 | … | … |
| No. areas of cropland/shrub and/or grass cover | >1 | 4 | 151 | 1.20 | 0.7456 |
| | 0 | 13 | 591 | … | … |
| Elevation (m)* | >272 | 77 | 78 | 1.01 | 0.3936 |
| | ≤272 | 298 | 306 | … | … |

*categorized by median value

of a river or stream within 5 km (OR 5.65; P=0.0050). No significant (P=0.3938) spatial autocorrelation (I=0.02) was detected for the model standardized residuals.

The risk of an outbreak in which other domestic poultry species were affected in addition to chickens, compared to outbreaks in which only chickens were affected, was associated (P<0.20) with epidemic phase, elevation >272 m, and the presence of a road, a lake or pond, a river or stream, an area of broadleaf tree cover, or area of cropland/shrub and/or grass cover within 5 km. The best fitting multivariate model of outbreak risk included the presence of a river or stream (OR 0.24, 95% CI, 0.11–0.53), an area of broadleaf tree cover (OR 0.30, 95% CI, 0.11–0.82), and an area of cropland/shrub and/or grass cover (OR 9.89, 95% CI, 1.12–86.9) within 5 km. No significant (P=0.2954) spatial autocorrelation (I=0.104) was detected for the model standardized residuals.

## Discussion

Few epidemiologic studies have been conducted on risk factors for outbreaks of highly pathogenic avian influenza. Most studies have focused on intensive, commercial poultry

systems (McQuiston et al. 2005, Thomas et al. 2005) and poultry farms (Rojanasatien et al. 2006). Some of the risk factors identified with a potential environmental component include sharing of water sources with other farms, open house systems, and presence of mammalian wildlife on the farm. Information on risk factors is needed so that control and prevention programs can be developed to limit the spread of HPAI, and in particular subtype H5N1 and the risk of zoonotic infections.

In this study, the presence of a primary or secondary road within 5 km of a village increased the risk of an outbreak of HPAI subtype H5N1 more than 5-fold. The presence of a major road close to a village might represent the mechanism of spread of influenza virus during the epidemic: it is likely (although impossible to conclusively prove) that HPAI subtype H5N1 spread between Romanian villages during 2005 and 2006 via the transport of infected poultry. Movement of contaminated poultry products and equipment might also have played a role. The most likely route of transportation is via primary and secondary roads, rather than minor roads, since the mean distance between all outbreak locations was 295 km. The importance of major roads as a risk factor for outbreaks highlights the need to implement and enforce movement restrictions when HPAI subtype H5N1 is first detected within national poultry flocks, to limit the size of the resulting epidemic, the impact on the national flock, and the risk of zoonotic transmission. Knowledge of the regular movement of poultry between localities within a country is key to anticipating how and over what distance an introduced disease might spread.

Based on the spatio-temporal occurrence of outbreaks, the Romanian epidemic of HPAI subtype H5N1 can be characterized by two phases (Ward et al. 2008): sporadic outbreaks occurring mostly in eastern and southern Romania during the autumn and winter of 2005, and a large epidemic occurring predominantly in central Romania during May and June, 2006. The presence of a primary or secondary road within 5 km of a village increased the risk of an outbreak of highly pathogenic avian influenza subtype H5N1 during the first phase more than 3-fold. However, proximity to a major road or a major road intersection was not included in the multivariate logistic regression best explaining the risk of outbreaks in the second phase of the epidemic. This result does not support the idea that outbreaks occurring in the latter part of the epidemic were caused exclusively by the illegal transportation of infected poultry. Alternatively, if illegal transportation of infected poultry contributed to spread during this second phase, such movements might not have been via major road routes. Another explanation for a change in the risk factors identified during different phases of the epidemic might be the influence of different interventions that were applied to control outbreaks, as the epidemic progressed.

The epidemic in Romania consisted of both primary and secondary outbreaks. Although it is likely that HPAI subtype H5N1 spread between Romanian villages during 2005 and 2006 via the transport of infected poultry (secondary cases), some outbreaks (primary cases) might also have been initiated by contact between infected wild bird species and domestic poultry. Overall, the presence of a river or stream within 5 km of a village increased the risk of an outbreak by nearly 2-fold, and increased risk during the second epidemic phase by 5−6 fold. Also, the presence of an area of flooded shrub or herbaceous land cover within 5 km increased outbreak risk during the first epidemic phase by >5-fold. A likely explanation for these associations is contact between domestic poultry and wild birds infected with HPAI subtype H5N1 within these environments.

Outbreaks of influenza in domestic poultry have been explained by contact with wild birds, based on the circumstantial evidence of co-location of wild birds and domestic poultry in time and space (de Benedictis et al. 2007; Normile 2005). HPAI subtype H5N1 virus is considered to have been introduced to Romania via migratory water fowl during the

autumn migration. The eastern part of Romania, and in particular the Danube River Delta, is an important location on the Siberian-West Africa migratory flyway. At least 23 species of *Anatidae* (ducks, geese and swans) overwinter in the Black Sea region (Waterbird Population Estimates 2006). Of these, at least 6 species have a breeding range that extends to West Siberia, including *Cygnus cygnus* (Whooper Swan), *Anas penelope* (Eurasian Wigeon), *Anas crecca* (Common Teal), *Anas acuta* (Northern Pintail), *Anas clypeata* (Northern Shoveler) and *Aythya marila* (Greater Scaup). HPAI subtype H5N1 virus has reportedly been isolated from the first 4 of these species in Romania (Ontanu et al. 2007). A high mortality rate was observed in swans in the Danube River delta during the epidemic. In this region of Romania, poultry production is almost entirely based at the village level. It is common for birds to have free range during the daylight hours, and contact with wild birds is likely. If the H5N1 subtype was introduced to Romania via migratory waterfowl, winter conditions might have initially limited its spread to southeastern Romania. Thus, only localized outbreaks occurred as the result of contact between wild birds and domestic village poultry at common congregation and feeding sites. Landscape features, such as rivers, streams and wetlands might represent the interface between wild birds and domestic poultry: areas where waterfowl reside, either permanently or temporarily (in the case of migratory species), and where village poultry species potentially can contact wild birds. Knowledge of the presence of such land cover could be used to develop risk maps for disease introduction (primary foci) that could be targeted in surveillance programs for HPAI.

The study of landscape, animal production and human factors in such epidemic transition zones can help us to understand why large outbreaks of HPAI subtype H5N1 occur in countries previously free of infection. Spatial statistics and geostatistical methods have a role to play in helping us resolve the debate over the role of migratory wild birds versus the movement of poultry and poultry products in the global spread of HPAI subtype H5N1.

**Acknowledgments** Support for the acquisition and analysis of the data used in this study was provided by the United States Department of Agriculture Foreign Agriculture Service.

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