Surveillance of Influenza Viruses in Waterfowl Used As Decoys in Andalusia, Spain

Estefanía Jurado-Tarifa1, Sebastian Napp2, Juan Manuel Gómez-Pacheco3, Manuel Fernández-Morente4, Juan Antonio Jaén-Téllez5, Antonio Arenas1, Ignacio García-Bocanegra1*

1 Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad de Córdoba-Agrofood Excellence International Campus, Córdoba, Spain, 2 Centre de Recerca en Sanitat Animal (CReSA), Universitat Autònoma de Barcelona-Institut de Recerca i Tecnologia Agralimentàries (UAB-IRTA), Bellaterra, Barcelona, Spain, 3 Laboratorio de Sanidad y Producción Animal, Consejería de Agricultura, Pesca y Desarrollo Rural de la Junta de Andalucía, Córdoba, Spain, 4 Servicio de Sanidad Animal, Consejería de Agricultura, Pesca y Desarrollo Rural de la Junta de Andalucía, Sevilla, Spain, 5 Agencia de Gestión Agraria y Pesquera de Andalucía, Consejería de Agricultura, Pesca y Desarrollo Rural de la Junta de Andalucía, Sevilla, Spain

Abstract

A longitudinal study was carried out to determine the seroprevalence of avian influenza viruses (AIVs) in waterfowl used as decoys in Andalusia, southern Spain. A total of 2319 aquatic birds from 193 flocks were analyzed before and after the hunting season 2011–2012. In the first sampling, 403 out of 2319 (18.0%, CI95%: 15.8–19.0) decoys showed antibodies against AIVs by ELISA. The AI seroprevalence was significantly higher in greese (21.0%) than in ducks (11.7%) (P<0.001). Besides, the spatial distribution of AIVs was not homogeneous as significant differences among regions were observed. The prevalence of antibodies against AIVs subtypes H5 and H7 were 1.1% and 0.3%, respectively, using hemagglutination inhibition test (HI). The overall and H5 seroprevalences slightly increased after the hunting period (to 19.2% and 1.4%, respectively), while the H7 seroprevalence remained at the same level (0.3%). The proportion of flocks infected by AIVs was 65.3%, while 11.2% and 4.9% of flocks were positive for H5 and H7, respectively. Viral shedding was not detected in any of the 47 samples positive by both ELISA and HI, tested by RRT-PCR. The individual incidence after the hunting season was 3.4%. The fact that S7 animals seroconverted, 15 of which were confirmed by HI (12 H5 and 3 H7), was indication of contact with AIVs during the hunting period. The results indicate that waterfowl used as decoys are frequently exposed to AIVs and may be potentially useful as sentinels for AIVs monitoring. The seroprevalence detected and the seropositivity against AIVs H5 and H7, suggest that decoys can act as reservoirs of AIVs, which may be of animal and public health concern.

Citation: Jurado-Tarifa E, Napp S, Gómez-Pacheco JM, Fernández-Morente M, Jaén-Téllez JA, et al. (2014) Surveillance of Influenza Viruses in Waterfowl Used As Decoys in Andalusia, Spain. PLoS ONE 9(6): e98890. doi:10.1371/journal.pone.0098890

Editor: James P. Stewart, University of Liverpool, United Kingdom

Received February 7, 2014; Accepted May 8, 2014; Published June 5, 2014

Copyright: © 2014 Jurado-Tarifa et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The following funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. This work was partially supported by Regional Ministry of Agriculture and Fisheries of the Government of Andalusia, Spain. No additional external funding received for this study.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: nacho.garcia@uco.es

Introduction

Avian influenza viruses (AIVs) are among the most important emerging zoonotic pathogens worldwide, affecting a wide variety of avian and mammalian species, including humans [1]. Most strains of AIVs are low pathogenic (LPAIVs), causing minimal disease in infected animals. However, the H5 and H7 subtypes have implications for public and animal health owing to their potential to mutate to highly pathogenic viruses (HPAIVs), inducing severe disease and high mortality [2]. Public health relevance of AIVs is highlighted by the fact that the H5N1 subtype has caused, up to January 2014, 650 human cases, of which 386 died [3]. It emerged in Southern China in the 1990s, but it was not until the winter of 2005/2006 that spread westward, mainly via migratory birds, reaching Central Asia, Europe and Africa [4]. In March 2013, a novel reassortant AIV (H7N9) was identified in China [5], and has caused, up to January 2014, 251 human cases with 56 deaths [3]. Whether this variant may reach the wild bird population in Europe is difficult to predict, so in this context it is essential to maintain the European Union wild bird surveillance [6]. Wild aquatic birds, especially Charadriiformes and Anseriformes, are considered natural reservoirs of AIVs, and do not usually develop clinical signs [2,7]. Waterfowl can play an important role in the transmission of LPAI and HPAI strains to poultry farms through long distances during migrations [8]. Besides, AIVs may also persist in the environment for long periods under appropriate conditions, favoring their transmission and maintenance between wild and domestic birds [9].

Due to the strategic location on the migratory flyway of wild birds between Eurasia and Africa, the high number of wetlands and the diversity of wild bird species, Spain is considered a risk area for HPAIVs introduction [10]. Since 2004, a National Avian Influenza Surveillance program has been implemented to determine the incidence of H5 and H7 subtypes of AIVs in wild and domestic birds in Spain. To date, four outbreaks of AIVs have been reported in this country. In 2006, H5N1 strain of HPAIV was detected in a Great Crested Grebe (Podiceps cristatus) found dead in the Basque Country (Northern of Spain) [11]. Two outbreaks were detected in poultry in 2009, one of an H5N3 strain of LPAIV in a duck meat production farm in the Community of Navarra (Northern Spain) [12], and the second one associated to an H7N7 strain of HPAIV in a laying hen farm in Guadalajara (Central Spain), next to a wetland with high density of wild birds.
Serological Analysis

The presence of antibodies against the nucleoprotein of AIVs type A was determined using a commercial blocked enzyme linked assay (ELISA) [10,FLUK.3, INGEZIM INFLUENZA A, Inge-nasa, Madrid, Spain] according to the manufacturer’s recommendations. Sera positive against AIVs type A were then analyzed by means hemagglutination inhibition test (HI) as previously described [21]. Sera that showed HI titers ≥1:16 were considered as positive. ELISA positive samples were tested for the detection of specific antibodies against H5 using inactivated antigens of both H5N3 (A/Teal/Eng/7394-2005/06) and H5N1 (A/Ck/Scot/59) strains. A serum was considered positive to H5 subtype when showed positive results against both H5N3 and H5N1. ELISA positive samples were also tested for the detection of specific antibodies against H7 using inactivated antigens of both H7N1 (A/Afr.Star/Eng/983/79) and H7N7 (A/Thy/Eng/647/77) strains. A serum was considered positive to H7 subtype when showed positive results against both H7N1 and H7N7. The strains used in the present study were provided by the European AIVs Reference Laboratory in Weybridge (United Kingdom). Hemagglutination inhibition tests were performed at the National AIVs Reference Laboratory in Algete (Madrid).

Virological Analysis

After serological results, a convenience sampling was carried out to detect AIVs shedding from birds positive by ELISA. A total of 47 cloacal swabs (34 from the first sampling and 13 from the second), from 44 selected seropositive birds by ELISA were collected. Nine of the 44 seropositive birds were also positive against H5 by HI test. Samples were analyzed using an one-step real time reverse transcription polymerase chain reaction (RRT-PCR) with Taqman specific for the matrix gene (gene M) in the segment 7 of AIVs using primers previously described [22]. Briefly, viral RNA was extracted by using BioSprint 96 DNA Blood Kit and poly A RNA carrier (Qiagen, Valencia, CA) following the manufacturer’s instructions. Amplification was performed using a one-step RT-PCR in ABI 7300 equipment (Applied Biosystems, Foster City, CA).

Statistical Analysis

The prevalence of antibodies against AIVs was estimated from the ratio of positive to the total number of samples, with the exact binomial confidence intervals of 95% [23]. Associations between the serological results and explanatory variables (municipality, region, species, census) were analyzed using a Generalized linear mixed-effects models with an underlying binomial distribution (log link). Models were fitted by Laplace approximation, implemented in the glmer function of the lme4 package for R [http://cran.r-project.org/package=lme4]. Inference was based on model comparison of nested models (ANOVA), and the process of model selection was based on the lowest Akaike information criterion (AIC) value. Statistical analyses were carried out in R software (http://www.r-project.org/).

Results

Antibodies against AIVs were found in 418 out of 2319 (18.0%, CI95%; 15.9–19.0) birds tested by ELISA during the first sampling (Table 1). The effect of clustering of animals within flocks was assessed by comparing the model with no explanatory variables (Table 2), but with flocks included as random effect (Model 1), with the model with no explanatory variables, and no random effects (Model 0). The lower value of AIC for Model 1 compared to Model 0, indicates that is important to account for the fact that
animals are clustered within flocks. The model with the lowest AIC, and therefore selected (Model 3) included flocks as random effect, and species and provinces as fixed effects. There were differences in seropositivity between geese (21.0%; 327/1557) and ducks (11.7%; 88/753). The results of the model (Table 2) indicate that seropositivity was significantly higher in geese as compared to ducks (OR = 2.5; 95%CI = 1.8–3.4). On the other hand, there were also differences in seropositivity among provinces: 6.1% in Huelva, 16.9% in Cadiz and 20.0% in Seville (Figure 1). The results of the model (Table 2) indicate that seropositivity was significantly higher in Cadiz (OR = 5.7; 95%CI = 1.4–25.3) and in Seville (OR = 8.4; 95%CI = 2.3–35.1) as compared to Huelva.

A total of 403 seropositive animals could be analyzed by the HI test (15 of the 418 samples had insufficient volume). The individual seroprevalence against H5 and H7 subtypes were 1.1% (25/2279; CI95%: 0.7–1.6) and 0.3% (8/2304; CI95%: 0.2–0.7), respectively (Table 1).

Ninety four out of 143 flocks (65.7%) had at least one positive animal by ELISA (Table 1). The seroprevalence within positive flocks ranged from 4.2 to 90.5% (mean = 25.3). Antibodies against H5 were detected in 16 of the 143 flocks (i.e. prevalence of 11.2%), while antibodies against H7 were detected in 7 of the 143 flocks (i.e. prevalence of 5.9%). The within flock seroprevalence ranged between 5.9% and 57.1% for H5, and between 5.9% and 33.3% for H7.

The individual seroprevalence increased slightly after the hunting season (to 19.2%; 406/2110). A total of 209 animals (three seropositive and 206 seronegative decoys in the first sampling) could not be sampled in the second sampling. Results for HI were similar to those obtained in the first sampling, with 28

Figure 1. Map of Andalusia (Southern Spain) showing the location of decoys sampled. The darker gray gradient represents the seroprevalence against AIVs in the different municipalities sampled. doi:10.1371/journal.pone.0098890.g001
birds serologically positive to H5 (1.4%; 28/2006) and seven to H7 (0.3%; 7/2006). However, 66 decoys positive in the first sampling showed negative results in the second sampling, while 57 individuals seroconverted after the hunting period (individual incidence of 3.4%); 12 of them were confirmed as positive against H5 and three against H7 by HI.

The flock prevalence after the hunting period (65.2%; 88/135) was very similar to that found in the first sampling (Table 1). However, while the prevalence of H5-positive flocks slightly increased (to 11.8%; 16/135), the prevalence of H7-positive flocks slightly decreased (to 4.4%; 6/135) in the second sampling. Three flocks seroconverted to H5, two of them with only one positive birds detected and the other one with five new seropositive individuals. In addition, a flock seroconverted to H7 after the hunting period. On the other hand, three flocks positive to H5 and two positive to H7 in the first sampling, were negative in the second sampling.

AIVs RNA was not detected in any of the 47 cloacal swabs analyzed by RRT-PCR, including three animals positive against H5.

Discussion

Even though wild and domestic birds have been the main target of surveillance programs and AI investigations, studies on the prevalence of AIVs birds reared in backyard are very limited in Europe [24] and, to the best of our knowledge; this is the first study on AIVs carried out in waterfowl used as decoys for hunting. The results indicate an enzootic circulation of AIVs in decoys in Southern Spain. Recent studies indicate that previous exposure to LPAIV confer some cross-protection, increasing the bird's probability of surviving HPAIV H5N1 infection, and theoretically, these surviving birds could contribute to the spread of the disease [8,13,25]. On the other hand, experimental studies have also shown that development of LPAIV antibodies can result in a reduced magnitude and duration of shedding when infected with other AIVs including HPAIV H5N1 [10,14,25], thereby decreasing the likelihood of further transmission.

Active circulation of AIVs has been previously detected in wild and domestic waterfowl in Spain (Table 3) [15,16,11,26–28], and highlights the role of these species in the epidemiology of AIVs [29,30]. Higher seropositivity was previously found in both wild (33%; 306/927) and domestic (40%; 131/331) birds from different regions of Andalusia [15]. In contrast, a lower overall seroprevalence (6.2%; 44/712) was detected in wild waterfowl from the Doñana National Park (southwestern Spain) [16]. Recently, the AIVs prevalence found in central and northeastern Spain from faeces and tracheal swab samples were 2.6% (37/1435) and 4.5% (62/1374), respectively [26,27] by means of rRT-PCR. A low prevalence (1.7%; 78/4578) was also found from fresh faeces in different Spanish wetlands using the same direct method [28]. Wide variations in prevalence of AIVs in wild birds have been also reported in other European countries (Table 3) [18,31–39,40–59]. The differences among studies could be due to the diagnosis methods, species analyzed, sample size, type of samples or environmental factors. The higher values obtained in the present study are logical considering that the technique used was an indirect method which detects antibodies against the nucleoprotein.

Table 1. Seroprevalences against AIVs in decoys in Andalusia (southern Spain) before and after the hunting season 2010–2011.

| Seroprevalence | % positive ELISA (number/overall) | % positive H5 (number/overall) | % positive H7 (number/overall) |
|----------------|----------------------------------|-----------------------------|-----------------------------|
| Individual     | First sampling                   | 18.0 (418/2319)             | 1.1 (25/2279)               | 0.3 (7/2304)               |
|                | Second sampling                  | 19.2 (406/2110)             | 1.4 (28/2006)               | 0.3 (7/2006)               |
| Flock          | First sampling                   | 65.7 (94/143)               | 11.2 (16/143)               | 4.9 (7/143)                |
|                | Second sampling                  | 65.2 (88/135)               | 11.8 (16/135)               | 4.4 (6/135)                |

doi:10.1371/journal.pone.0098890.t001

Table 2. Results of model selection process.

| Model            | Random effects | Random effects | Random effects | Random effects |
|------------------|----------------|----------------|----------------|----------------|
| No fixed effects | No fixed effects | Fixed effect: species | Fixed effects: species + provinces |
| AIC              | 2187           | 1908           | 1873           | 1866           |

Random effects (Flock)

| Variance | 2.2 | 2.3 | 2.1 |

Fixed effects

| Intercept | −2.2 | −2.8 | −4.7 |
| Geese OR (95%CI) | 2.5 (1.8–3.4) | 2.5 (1.8–3.4) |
| Cadiz OR (95%CI) | 5.7 (1.4–25.3) |
| Seville OR (95%CI) | 8.4 (2.3–35.1) |

1Ducks as the reference category;
2Huelva as the reference category.

doi:10.1371/journal.pone.0098890.t002
tein of AIVs type A. Detectable levels of antibodies against AIVs appear one to two weeks after infection and can last for several months, which also allowed the detection of birds that were infected prior to the sampling period. Owing to the intermittent viral excretion, direct diagnostic methods may result in the underestimation of AIVs prevalence [60]. In fact, faecal shedding of AIVs was not found in any of the 44 seropositive animals examined in the present study. Taking into account that fecal shedding of the virus is in general of less than 11–15 days [60–62], the possibility of detecting the virus was low. Our results are consistent with previous studies and suggest that surveillance for virus shedding alone may provide incomplete information on the transmission potential relative to surveys which also include detection of antibodies [25,63]. Indirect techniques such as ELISA have potentially valuable applications for AIVs monitoring in bird species and should be considered when deciphering patterns of exposure, differential infection, and rates of AIVs transmission [25,64].

Even though most of the decoy flocks analyzed (70.6%) were mixed, including geese and ducks, and individuals were bred with similar management conditions, a significantly higher seroprevalence was found in geese compared to ducks. Differences in the prevalence of AIVs among species have been previously described and could be associated to variations in the immunological response, behavior, density-related patterns or gregariousness patterns. In the majority of studies [26,27,30,31,33,36,59], the highest prevalences were detected in ducks. The dabbling behaviour of some ducks seems to play an important role in their

Table 3. Prevalence of AIVs in wild birds in Europe.

| Location     | N  | POSITIVE (%) | Type of test | Reference          |
|--------------|----|--------------|--------------|--------------------|
| Spain        | 208| 43           | ELISA/IH     | Arenas et al., 1990|
| Spain        | 712| 6.2          | ELISA        | Astorga et al., 1993|
| Spain        | 3500| 8            | RRT-PCR      | Barral et al., 2008|
| Spain        | 686| 7.9          | RRT-PCR      | Busquets et al., 2010|
| Spain        | 628| 4.6          | RT-PCR       | Pérez-Ramírez et al., 2010|
| Spain        | 4572| 1.7         | RRT-PCR      | Pérez-Ramírez et al., 2012|
| Portugal     | 3561| 2.3         | RT-PCR       | Henriques et al., 2011|
| Portugal     | 1542| 4.5         | RRT-PCR      | Tolf et al., 2012|
| Italy        | 1039| 52.2        | ELISA/IH     | De Marco et al., 2004|
| Italy        | 326 | 66          | ELISA/IH     | De Marco et al., 2005|
| Italy        | 3000| 5           | RRT-PCR      | Cattoli et al., 2007|
| Italy        | 4083| 8           | RRT-PCR      | Terregino et al., 2007|
| Italy        | 147 | 0/1,3       | RT-PCR/ELISA | Delogu et al., 2012|
| Italy        | 2023| 2.2         | RT-PCR       | Kelvin et al., 2012|
| France       | 799 | 6.9         | RT-PCR       | Lebarbenchon et al., 2009|
| France       | 2901| 5.4         | RT-PCR       | Lebarbenchon et al., 2010|
| France       | 205 | 15          | RT-PCR       | Vittecoq et al., 2012|
| Belgium      | 7500| 0.02        | RT-PCR       | Marché et al., 2013|
| Germany      | 5864| 3.7         | RRT-PCR      | Rabl et al., 2009|
| Germany      | 1402| 1.07        | RRT-PCR      | Pannwitz et al., 2009|
| Germany      | 12652| 2.3      | RRT-PCR      | Lang et al., 2010|
| Netherlands  | 132 | 51.5        | ELISA        | Verhagen et al., 2012|
| Denmark      | 1381| 3.1         | RT-PCR       | Bragstad et al., 2007|
| Switzerland  | 2000| 4           | RT-PCR       | Baumer et al., 2010|
| Sweden       | 4800| 12.5        | RT-PCR       | Wallensten et al., 2007|
| Sweden       | 7728| 13.1        | RRT-PCR      | Latorre-Margalef et al., 2013|
| Norway       | 604 | 13.2        | RT-PCR       | Jonassen & Handeland, 2007|
| Norway       | 1529| 12.5        | RT-PCR       | Germundsson et al., 2010|
| Norway       | 2417| 15.5        | RRT-PCR      | Tannnessen et al., 2013|
| Austria      | 3151| 3.77        | RT-PCR       | Fink et al., 2010|
| Georgia      | 8343| 1.6         | RT-PCR       | Lewis et al., 2013|
| Turkey       | 402 | 0.49        | RRT-PCR      | Albayrak et al., 2010|
| Slovenia     | 2547| 4.4         | PCR          | Slavec et al., 2012|
| Finland      | 310 | 1.6         | ELISA        | Lindh et al., 2008|
| Northern Europe | 8500| 1         | RT-PCR       | Fouchier et al., 2003|
| Northern Europe | 36809| 2.6    | RT-PCR       | Munster et al., 2007|

doi:10.1371/journal.pone.0098890.t003
higher prevalences as the AIVs excreted in faeces remain in surface waters and are ingested by other ducks, while geese graze in pastures and agricultural fields [59]. However, in North America and Alaska, among hunter-harvested species, the greater white-fronted goose (Anser albifrons) and Emperor goose (Anser canagica) had the highest prevalences, respectively [25,65]. A higher susceptibility to AIVs infection was found in geese compared to swans [66], while ducks are considered more susceptible than chickens [67]. Additional studies would be needed to explain these differences in prevalence among species.

The flock seroprevalence levels indicate widespread circulation of AIVs in decoys in Andalusia. However, the results show that the spatial distribution of AIVs in decoys in Andalusia was not homogeneous. Statistically significant differences in seroprevalence were observed among municipalities, with highest seroprevalences in the regions located close to the river Guadalquivir (Cádiz and Seville), the largest river in Andalusia. The seropositivity obtained in Huelva (6.1%) was similar to that previously detected by Astorga et al. (1994) in Doñana National Park, the main wetland in Spain, and within the route of different migratory species between Europe and Africa. Environmental factors have been associated with the risk of AIVs transmission [9,68]. In Andalusia, 36 of the 41 (85.4%) hunting areas for aquatic birds (total area: 47986 ha) are located in the Seville province (42973ha), and a 93.7% of the 25720 birds hunted in 2012 were obtained in this province [69]. In this sense, the risk of contact between decoys and wild birds may increase in flocks close to wetlands, especially in decoy flocks reared outdoors.

The individual incidence between the first and the second sampling was 3.4%. A total of 57 individuals seroconverted, which confirms AIVs infections during the study period. The results may be associated to contact with infected decoys, migratory and resident infected wild birds or to the presence of AIVs in the environment where decoys are kept during the hunting activity [70]. The second sampling was carried out just after the winter period coinciding with the greatest presence of migratory birds in Spain. Studies carried out in this and other Mediterranean countries have shown that wintering grounds favor the introduction of new AIVs strains and their transmission among resident birds [27,36,71].

The detection of antibodies against H5 and H7 by HI indicates that birds were exposed and responded serologically to the contact with these AIVs subtypes. In fact, 15 out of the 57 seroconverted decoys analyzed in the second sampling were confirmed as H1 positive against H5 (12) and H7 (3), indicating recent infections with both subtypes. Although the pathogenicity of the circulating strains could not be determined, the results indicate that decoys could represent a risk for HPAIVs emergence associated to genetic mutations, as has been previously demonstrated [30]. The fact that H5 and H7 are circulating in the area, may result in the introduction of these subtypes into poultry and the development of LPAIV or even HPAIV outbreaks, as the introduction of AIVs into poultry are primarily the result of wild bird activity, not only as a consequence of direct contact, but also indirectly via contaminated water [9]. The seropositivities to H5 and H7 subtypes found are in accordance with those previously reported in central and northern Spain by RRT-PCR [11,26,27], which suggests a limited circulation of both subtypes in Spain. Limited H5 and H7 subtypes circulation have been also found in other European countries [18,34,55,72]. LPAIVs circulation has been frequently detected in the Iberian Peninsula, being the H3N8, the H4N6 and the H1N1 the most common subtypes detected in Spain [26,28], and the H10N7, the H9N2 and the H11N3 in Portugal [32].

In conclusion, the results of the present study confirm a widespread circulation of AIVs in waterfowl species used as decoys in Andalusia. The seroprevalence obtained indicates that decoys are frequently exposed to AIVs and may potentially be useful as sentinels for AIVs monitoring. The seropositivity against AIVs H5 and H7, suggest that decoys can act as reservoirs of these subtypes, which may be of animal and public health concern. Because of the direct contact among decoys, wildlife and human, these species should be considered as risk species for the transmission of bird-borne pathogens, including AIVs. Control measures to limit transmission from wild birds to decoys as well as from decoys to wild birds should be implemented.

Acknowledgments

We would like to thank the Laboratories of the Regional Ministry of Agriculture and Fisheries and Agencia de Gestión Agraria y Pesca de Andalucía (AGAPA) of the Government of Andalusia for providing samples and technical support. We thank Amparo Cabezas in the data collection. We gratefully acknowledge the assistance of the Central Veterinary Laboratory in Algete, Madrid (MAPA), and especially to Azucena Sánchez, with the hemagglutination inhibition assays.

Author Contributions

Conceived and designed the experiments: IGB EJ MF JAJ AA. Performed the experiments: IGB MF JAJ. Analyzed the data: EJ MF JAJ. Wrote the paper: IGB MF JAJ AA. Drafted the final manuscript: EJ MF JAJ AA IGB.

References

1. Jeong-Ki K, Negoveticha NJ, Forresta HL, Webster RG (2009) Ducks: The “Trojan Horses” of H5N1 influenza. Influenza Other Respir Viruses 3(4): 121–128.
2. Alexander DJ (2000) A review of avian influenza in different bird species. Vet Microbiol 74: 3–13.
3. World Health Organization (2014) Available: http://www.who.int/influenza/en/ Accessed 27 January 2014.
4. Gilbert M, Xiao X, Péiffer DU, Epprecht M, Boles S (2008) Mapping H5N1 highly pathogenic avian influenza risk in Southeast Asia. Proc Natl Acad Sci U S A 105(12): 4769–74.
5. Nicoll A, Danielson N (2013) A novel reassortant avian influenza A (H7N9) virus in China - what are the implications for Europe. Euro Surveill 18(10): 20452.
6. Schenk C, Plachouras D, Danielsson N, Nicoll A, Robyens E, et al. (2013) Outbreak with a novel avian influenza A(H7N9) virus in China—scenarios and triggers for assessing risks and planning responses in the European Union, May 2013. Euro Surveill 18(20).
7. Sturm-Ramirez KM, Hulse-Post DJ, Govorkova EA, Hummerd J, Seiler P, et al. (2005) Are Ducks Contributing to the Emergence of Highly Pathogenic H5N1 Influenza Virus in Asia?. J Virol 79(17): 11269–11279.
8. Kevaurohaen J, van Riel D, van Amerongen G, Bestebroer T, Beyer WE, et al. (2008) Wild Ducks as Long-Distance Vectors of Highly Pathogenic Avian Influenza Virus (H5N1). Emer Infect Dis 14(4): 600–606.
9. Alexander DJ (2007) An overview of the epidemiology of avian influenza. Vaccine 25: 5637–5644.
10. Martinez M, Muñoz MJ, De la Torre A, Iglesias I, Peris S, et al. (2009) Risk of introduction of H5N1 HPAI from Europe to Spain by Wild Water Birds in Autumn. Transbound Emerg Dis 56(3): 86–98.
11. Barral M, Alvarez V, Juste RA, Aguirre I, Inchausti I (2008) First case of highly pathogenic H5N1 avian influenza virus in Spain. BMC Vet Res 4: 50.
12. RASVE (2009) Ministerio de Medio Ambiente Medio Rural y Marino. Available: http://rasve.magrama.es/RASVE_2008/Publica/Focos/Consulta.aspx Accessed 16 January 2014.
13. Iglesias I, Martinez M, Muñoz MJ, de la Torre A, Sánchez-Vizcaíno JM (2010) First Case of Highly Pathogenic Avian Influenza in Poultry in Spain. Transbound Emerg Dis 57(4): 203–203.
14. RASVE (2013) Ministerio de Medio Ambiente Medio Rural y Marino. Available: http://rasve.magrama.es/RASVE_2008/Publica/Focos/Consulta.aspx Accessed 16 January 2014.
15. Arenas A, Carranza J, Perera A, Miranda A, Maldonado A, et al. (1990) Type A influenza viruses in birds in southern Spain: serological survey by enzyme-linked immunosorbent assay and haemagglutination inhibition tests. Avian Pathol 19(3): 539-546.

16. Astorga RJ, Leiva-L, Cabero M J, Arenas A, Maldonado A, et al. (1994) Avian Influenza in wild waterfowl and shorebirds in the Donana National Park. Serological survey using enzyme-linked immunosorbent assay. Avian Pathol 23(2): 339-344.

17. Vieitesco M, Grandhomme V, Gammelin M, Crescenzo-17. Tsiodras S, Kelesidis T, Kelesidis I, Bauchinger U, Falagas ME (2008) Human

18. Lebarbenchon C, Albespy F, Brochet A-L, Grandhomme V, Renaud F, et al. (2013) Influenza A virus surveillance in wild birds in Bavaria: occurrence and heterogeneity of H5 and N1 encoding genes. Zoonoses Public Health 57(4-5): 184–194.

19. Verhagen HJ, Munster VJ, Moss J, Lexmond P, Vuong O, et al. (2012) Avian Influenza A Virus in Wild Birds in Highly Urbanized Areas. PLoS ONE 7(6): e37299.

20. Bragstad K, Jorgensen PH, Handberg K, Hammer AS, Kabell S, et al. (2007) Molecular and epidemiological characterization of avian influenza viruses in wild and domestic birds in Denmark, Northern Europe. Virol J 4: 43.

21. Lebarbenchon C, Brown JD, Luttrell MP, Stallknecht DE (2012) Comparison of high pathogenic avian influenza viruses in wild and domestic birds in Denmark, Northern Europe. Virol J 4: 136–142.

22. Terregino C, De Nardi R, Guberti V, Scremin M, Raffini E, et al. (2007) Active surveillance for highly pathogenic Avian Influenza A Virus in migratory birds. PLoS ONE 2(11): e10794.

23. Cattoli G, Terregino C, Guberti V, De Nardi R, Drago A, et al. (2007) Influenza A virus surveillance in wild birds in Central and Eastern Europe. PLoS ONE 2(11): e10794.

24. Cattoli G, Terregino C, Guberti V, De Nardi R, Drago A, et al. (2007) Influenza A virus subtypes in wild birds in North-Eastern Spain (Catalonia). Virus Res 138(1-2): 228–234.

25. Kelly AA, Meloni D, Sansonetti P, Borghetto I, Row T, et al. (2012) Influenza A Virus Infection in Wild Birds by Analysis of Avian Fecal Samples from the Environment. J Wildl Dis 48(4): 512–518.

26. Lang V, Rinder M, Hafner-Marx A, Rahl S, Bogner KH, et al. (2010) Avian influenza A virus monitoring in wild birds in Bavaria: occurrence and heterogeneity of H5 and N1 encoding genes. Zoonoses Public Health 57(4-5): 184–194.

27. Perez-Ramirez E, Gerrikagoitia X, Barral M, Hofle U (2010) Detection of low pathogenic avian influenza viruses in wild birds in Southern Spain: serological survey by enzyme-linked immunosorbent assay. Avian Pathol 39(4): 339–344.

28. Olsen B, Munster VJ, WP Waldenstroem J, Wallensten A, et al. (2006) Spread of Avian Influenza Viruses by Common Teal (Anas crecca) in Migratory Birds. PLoS ONE 1(1): e186.

29. Perez-Ramirez E, Gerrikagoitia X, Barral M, Hofle U (2010) Detection of low pathogenic avian influenza viruses in wild birds in Castilla-La Mancha (south central Spain). Vet Microbiol 146(3-4): 200-208.

30. Perez-Ramirez E, Acvedo P, Alipua A, Gerrikagoitia X, Alba A, et al. (2012) Ecological Factors Driving Avian Influenza Virus Dynamics in Spanish Wetland Ecosystems. PLoS ONE 7(1): e24611.

31. Webster RG, Hulse DJ (2004) Microbial adaptation and change: avian influenza. Rev Sci Tech 23(2): 453-63.

32. Olsen B, Munster VJ, Wallensten A, Waldenstrom P, et al. (2004) Multiyear surveillance of influenza A virus in wild birds in Portugal. Avian Pathol 33(5): 480–485.

33. De Marco MA, Fonê E, Campitelli L, Doloys M, Raffini E, et al. (2005) Influenza virus circulation in wild aquatic birds in Italy during H5N2 and H7N1 epidemic periods (1998 to 2000). Avian Pathol 33(5): 480–485.

34. De Marco MA, Fonê E, Campitelli L, Doloys M, Raffini E, et al. (2005) Influenza virus circulation in wild aquatic birds in Italy during H5N2 and H7N1 epidemic periods (1998 to 2000). Avian Pathol 33(5): 480–485.

35. Lebarbenchon C, Brown JD, Luttrell MP, Stallknecht DE (2012) High Influenza A virus infection rates in mallards bred for hunting in the Camargue, South of France. PLoS ONE 7(1): e37299.

36. Terregino C, De Nardi R, Guberti V, Scremin M, Raffini E, et al. (2007) Influenza A Virus Infection in Wild Birds by Analysis of Avian Fecal Samples from the Environment. J Wildl Dis 43(4): 512–518.

37. Lang V, Rinder M, Hafner-Marx A, Rahl S, Bogner KH, et al. (2010) Avian influenza A virus monitoring in wild birds in Bavaria: occurrence and heterogeneity of H5 and N1 encoding genes. Zoonoses Public Health 57(4-5): 184–194.

38. Kelvin AA, Meloni D, Sansonetti P, Borghetto I, Row T, et al. (2012) Influenza A Virus Infection in Wild Birds by Analysis of Avian Fecal Samples from the Environment. J Wildl Dis 48(4): 512–518.

39. Concejero de Medio Ambiente y Ordenacion del Territorio. Junta de Andalucia (2012) Available: http://www.juntadeandalucia.es/medioambiente/site/ppc/Accesed 21 January 2012.
70. Nielsen AA, Jensen TH, Stockmarr A, Jørgensen PH (2013) Persistence of low-pathogenic H5N7 and H7N1 avian influenza subtypes in filtered natural waters. Vet Microbiol 166: 419–428.

71. Alba A, Bicout DJ, Vidal F, Carcó A, Allepuz A, et al. (2012) Model to Track Wild Birds for Avian Influenza by Means of Population Dynamics and Surveillance Information. PLoS ONE 7(8): e44354.

72. European Commission website. Health and consumer. Available: http://ec.europa.eu/food/animal/diseases/controlmeasures/avian/eu_resp_surveillance_en.htm. Accessed 14 January 2014.