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Calogero Terregino, Roberta de Nardi, Vittorio Guberti, Mara Scremin, Elisabetta Raffini, et al.. Active surveillance for Avian Influenza viruses in wild birds and backyard flocks in Northern Italy during 2004-2006. Avian Pathology, 2007, 36 (04), pp.337-344. 10.1080/03079450701488345 . hal-00540088

HAL Id: hal-00540088

<https://hal.science/hal-00540088>

Submitted on 26 Nov 2010

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Journal:	<i>Avian Pathology</i>
Manuscript ID:	CAVP-2006-0195.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	09-Feb-2007
Complete List of Authors:	<p>Terregino, Calogero; OI/FAO and National reference laboratory for Avian Influenza and Newcastle disease, Virology Laboratory, Virology Laboratory</p> <p>De Nardi, Roberta; OI/FAO and National reference laboratory for Avian Influenza and Newcastle disease, Virology Laboratory</p> <p>Guberti, Vittorio; Istituto Nazionale per la Fauna Selvatica Â A. GhigiÂ</p> <p>Scremin, Mara; Istituto Nazionale per la Fauna Selvatica Â A. GhigiÂ</p> <p>Raffini, Elisabetta; Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna, Sezione di Ravenna</p> <p>Moreno Martin, Ana; Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna, Sede di Brescia</p> <p>Cattoli, Giovanni; OI/FAO and National reference laboratory for Avian Influenza and Newcastle disease, Virology Laboratory</p> <p>Bonfanti, Lebara; Istituto Zooprofilattico Sperimentale delle Venezie, Centro Regionale di Epidemiologia Veterinaria (CREV)</p> <p>Capua, Ilaria; OI/FAO and National reference laboratory for Avian Influenza and Newcastle disease, Virology Laboratory</p>
Keywords:	avian influenza, surveillance, wild birds, backyard flocks

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Cavp-2006-0195.R1

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Short Title : Surveillance for Avian Influenza in Italy

Received: 28 December 2006

Cavp-2006-0195.R1

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Abstract

Following the avian influenza (AI) epidemics that occurred in Italy between 1997 and 2003, the Italian Ministry of Health in collaboration with veterinary authorities promoted, funded and implemented a national surveillance programme. The main objectives of the surveillance effort were to identify avian influenza viruses (AIVs) circulating in wild birds and to investigate the role of backyard poultry flocks in the dynamics of infection in a densely populated poultry area (DPPA). Over two years (2004-2006) 164 backyard flocks and 4,083 wild birds (mainly migratory *Anseriformes* and *Charadriiformes*) were sampled in three regions in the North of Italy. Samples collected were screened by means of real time reverse transcriptase-polymerase chain reaction (RRT-PCR) and the positive samples were processed for attempted virus isolation in embryonated fowl's specific pathogen free (SPF) eggs.

At the end of the study period 27 low pathogenic avian influenza (LPAI) viruses had been isolated from backyard flocks and 49 strains obtained from wild birds. Of these 26 belonged to the H5 or H7 subtype and were closely related to contemporary low pathogenic strains of Eurasian lineage. The findings confirm that backyard free range farming is at high risk for AIVs introduction, and the role of wild waterfowl in the introduction and perpetuation of LPAI viruses during the winter season in Southern Europe.

Comment [T1]: Eliminate were

Introduction

Prior to the ongoing H5N1 avian influenza (AI) panzootic, Italy had been affected by several incursions of AI viruses of the H5 and H7 subtypes (Capua *et al.*, 1999; Capua & Marangon, 2000; Capua *et al.*, 2003). The consequences of these outbreaks were very severe for the poultry industry, and for this reason Italian veterinary authorities implemented a nation-wide surveillance programme beginning in 2004.

This programme is still ongoing, and has revealed itself useful in understanding better the complex ecology of AI viruses (AIVs) and has had a positive effect on the public opinion. In particular such efforts are perceived as actions taken by public health authorities against the westward spread of the Asian H5N1 virus (Munster *et al.*, 2006; Gilbert *et al.*, 2006; Olsen *et al.*, 2006).

Comment [T2]: Eliminate in the meantime

The main objectives of the surveillance effort were to identify any AIVs circulating in wild birds and to establish whether these viruses were also infecting backyard poultry, which are thought to act as a link between the wild host and intensively reared poultry. The surveillance effort was focussed in North Eastern Italy, as this is where AI epidemics had occurred in the past, and incidentally has a unique combination of densely populated poultry areas (DPPAs) and wetlands.

The wetlands are located along the Eurasian Anatidae flyway, and therefore represent resting and wintering sites for many wild ducks and geese migrating south from Northern and Eastern Europe. In addition, waders, migrating south stop to rest and feed in the same wetlands, before undertaking the long flight over the Mediterranean Sea that precedes their over-wintering in Africa. The migrant population includes a high proportion of juveniles, hatched during the previous spring. Young birds are known to be highly susceptible to influenza virus infection (Cavanagh *et al.*, 1980). Therefore, during the winter season, thousands of susceptible migratory birds originating from different sites in Northern and

Comment [T3]: Eliminate also

Eastern Europe congregate in Italian wetlands and intermingle. This situation represents an ideal ecological setting for the transmission and perpetuation of AIVs.

Materials and methods

Specimens from backyard flocks. Between September 2004 to March 2006 a total of 164 backyard flocks in three regions Lombardia, Veneto and Emilia-Romagna (Figure 1a), were selected in areas at risk of infection. The number and location of backyard flocks to be sampled were identified by regional veterinary epidemiological centres on the basis of risk factors such as proximity of the farm with wetlands or DPPA and rearing of mixed species in the open. The sampling scheme was developed with the criterion that in case of infection at least one positive farm would be detected taking into account an estimated prevalence of 5% with a confidence level of 95%.

All selected backyard flocks raised primarily waterfowl. Commonly reared species were: mallard (*Anas platyrhynchos*), Pekin duck (*Anas platyrhynchos* f. dom.), muscovy duck (*Cairina moschata*) and goose (*Anser anser* f. dom). Often, *Galliformes* such as chickens (*Gallus gallus*), turkeys (*Meleagris gallopavo*) and guinea fowl (*Numida meleagris*) were also present in the same flock. The surveillance programme was designed to allow monitoring of the same farm approximately every 45 days. In these flocks a minimum of 10 cloacal swabs from each representative species (20 in farms with more than 250 birds or from all birds if less than 10), and at least one sample of fresh dropping from ducks and/or geese were collected for AI detection. The cloacal swabs (pool of 5 samples) and faecal samples (single or pooled) were analysed directly by virus isolation in embryonated chicken eggs without performing any preliminary screening tests.

Specimens from wild birds. During the winter seasons 2004/2005 and 2005/2006 4,083 cloacal swabs from wild birds were collected from 38 different species present in different wetlands of Northern Italy (Lagoon of Venice, Salt marsh of Cervia in Emilia-Romagna region, and the Po river delta). The samples were collected from trapped birds in bird ringing stations or from birds shot during normal hunting activities.

The species sampled were mainly migratory *Anseriformes* and *Charadriiformes* (Table 1) that fly into Italy from North and East European countries (Figures 2a and 2b). For the sake of clarity, the main collection sites have been named North (N), South (S) and West (W) sampling areas (Figure 1b) according to their geographic location in the study area. The collection sites include the most important wintering areas in North Italy. The geographical vicinity of the collection sites to the densely populated poultry area (DPPA) in Northern Italy is presented in Figures 1a and 1b.

Cloacal swabs were collected by trained personnel (gamekeepers, bird census takers, ornithologists, veterinarians) with a cotton swabs and were stored in transport media composed of phosphate-buffered saline (PBS)/glycerol (9/1) containing antibiotics (10,000 U/mL of penicillin, 10 mg/mL of streptomycin, 0.25 mg/mL of gentamycin and 5,000 U/mL of mycostatin). These samples were taken to the diagnostic laboratory within 24-48 hours in a refrigerated container (+2 - +8°C) and stored at -80°C until analysed.

RNA detection and virus isolation. The samples collected from wild birds were screened by means of real time reverse transcriptase-polymerase chain reaction (RRT-PCR) specific for type A influenza viruses (Spackman *et al.*, 2002; Cattoli *et al.*, 2004). Viral RNA was extracted from allantoic fluid using the High Pure TM RNA Isolation Kit (Roche®). Samples with threshold cycle value ≤ 35 were considered positive for Influenza A viral RNA and were subsequently processed for virus isolation using standard methods. Briefly, 100 μ l of the original sample were inoculated into the allantoic cavity of 9-11-day-old embryonating

specific pathogen free (SPF) eggs for virus isolation attempts according to EU Directive 92/40 (CEC, 1992). Haemagglutinating isolates were characterised by means of the haemagglutination inhibition tests (HI) and neuraminidase inhibition (NI) tests using specific hyperimmune chicken antisera to the reference strains of influenza virus (Alexander & Spackman, 1981). Isolates belonging to the H5 and H7 subtypes were pathotyped by sequencing the haemagglutinin gene segment (Hoffman *et al.*, 2001). Sequences were obtained using the BigDye Terminator v 3.1 cycle sequencing kit (Applied Biosystems) in a 3100-Avant Genetic Analyzer (Applied Biosystems).

The nucleotide sequences obtained were aligned by Clustal W and evolutionary distances were calculated by Kimura's Two-parameter method (Nei & Kumar, 2000). Phylogenetic dendrograms were constructed using the Neighbor Joining (NJ) method and bootstrap analysis (1,000 replicates) by MEGA 3 software (Kumar *et al.*, 2004; Nei & Kumar, 2000). Branches with bootstrapping values ≥ 70 were considered significant, corresponding to a confidence interval $\geq 95\%$ (Hillis & Bull, 1993).

Accession numbers. The sequences of the haemagglutinin gene segments of representative H5/H7 strains have been deposited in GenBank with the following accession numbers:

- A/mallard/Italy/3401/05 (H5N1) **DQ838508**
- A/goose/Italy/4692-11/04 (H7N7) **DQ838511**
- A/mallard/Italy/4234/04 (H7N7) **DQ838510**
- A/teal/Italy/3812/05 (H5N3) **DQ838509**
- A/duck/Italy/4692-30/04 (H7N7) **DQ838512**
- A/mallard/Italy/4776-36/04 (H7N7) **DQ838513**
- A/mallard/Italy/4810-7/04 (H7N7) **DQ838514**
- A/mallard/Italy/4810-79/04 (H7N4) **DQ838515**

- A/teal/Italy/3931/2005 (H5N2)

DQ851561

Results

Over the study period 27 AIVs were isolated from 19 of 164 backyard flocks tested (11.6%). Apart from one virus isolated from a guinea fowl, all isolates were obtained from domestic *Anseriformes* (Table 2). Among the isolates we obtained only three viruses belonging to H5 or H7 subtype namely an H5N3 virus of low pathogenicity (LP) AI and two H7N7 LPAI isolates.

The majority of viruses isolated (20/27) from backyard flocks were obtained from samples collected during the autumn/winter season. Only 7 positive samples were collected in the spring/summer season.

Of the 4,083 wild birds tested, 327 were positive for influenza type A by RRT-PCR (Table 1). Following virus isolation attempts, 47 AIVs belonging to 15 different subtype combinations (H1N1, H1N3, H3N8, H4N6, H5N1, H5N2, H5N3, H6N2, H7N4, H7N7, H9N2, H10N4, H10N7, H10N8, H11N9) were obtained from migratory waterfowl belonging to the Order *Anseriformes* (Table 3). One AI virus (AIV) of the H10N7 subtype was isolated from a wintering common snipe (*Gallinago gallinago*) in a hunting territory located in the western part of the study area. This appears to be the first isolation of an AIV from a wader in Italy. Another unexpected finding was the isolation of a virus of H6N2 subtype from a greater flamingo (*Phoenicopterus roseus*).

Comment [T4]: Eliminate the

No highly pathogenic (HP) AI viruses were detected.

Phylogenetic dendrograms were constructed for viral isolates of the H7 and H5 subtypes in order to assess their relationship with the viruses of the same subtypes causing outbreaks in domestic poultry in Europe in the recent past (Figure 3 and 4). With reference to

sequence and phylogenetic analysis of the haemagglutinin gene of H7 viruses isolated from wild birds, two separate sublineages, could be identified. These formed a larger cluster with the 13 viruses causing the recent LPAI outbreak in Italy.

Comment [T5]: Eliminate low pathogenic

It is interesting to point out that H7 isolates collected in domestic and wild waterfowl at the end of 2004 (see strains in the oval in Figure 3), are closely related showing a high homology at the nucleotide level (up to 99.3%).

The H7 isolates from wild birds were quite distinct from the H7 viruses responsible for the last two severe HPAI outbreaks in Europe (homology < 96.8% with the Italian strain H7N1 and the Dutch strain H7N7).

Similarly, the H5 viruses isolated from wild birds in 2005 clustered together in a group that is clearly distinct from the HPAI H5 viruses that caused outbreaks in 1997 and 1998 in domestic poultry in Italy and the contemporary HPAI H5N1 circulating in wild birds in Europe, Africa and Asia. (Figure 4). This clustering was supported by a high bootstrap value (100).

The LPAI H5 viruses were instead closely related to LPAI isolates from wild ducks sampled recently in North and Central European countries, in particular with the German strain A/mallard/Bavaria/1/2005 (H5N2) (nucleotide homology > 98%).

Discussion

This study confirms previous data (Campitelli *et al.*, 2004; De Marco *et al.*, 2005; Terregino *et al.*, 2005) indicating the active circulation of influenza A viruses in wild waterfowl in Italy, and highlights the role of wild and domestic waterfowl in the perpetuation of LPAI in nature (Webster & Hulse, 2004). In detail, certain species of wild waterfowl such as mallards, teals, pintails and wigeons should be considered as targets for sampling as they have been shown to

harbour the majority of AIVs obtained during this study. The greater susceptibility of these species to AI infection has also been reported in other studies (Olsen *et al.*, 2006).

The 49 strains isolated from wildfowl indicate that Italian wetlands represent, particularly during winter, an ecosystem which is able to perpetuate AIVs efficiently. The geographical vicinity between the wetlands and DPPAs suggest that they represent a risk for industrial poultry.

In comparing this data to others collected in Europe, it appears that the RRT-PCR method used in our study is more sensitive than other systems such as RT-PCR. In fact, in our study 15% of total samples were positive in RRT-PCR while in other studies a prevalence ranging from 1 to 2.3% were detected by RT-PCR (Fouchier *et al.*, 2003; Munster *et al.*, 2005).

However, given the low isolation rate (approximately 15%) of RRT-PCR positives and the overall virus isolation rates of AIVs from wild birds, it may seem reasonable to improve the outcome of isolation attempts. Possibly the quality of the specimen or the transportation medium or storage temperature and conditions could be improved. However, an additional possible explanation for the paucity of virus isolates obtained could be related to the level and duration of viral shedding in these birds once they arrive in Italy. It appears that influenza virus detection can be very high in Northern Europe prior to the winter migration (Olsen *et al.*, 2006; Okazaki *et al.*, 2000) and it is possible that a progressive decrease of shedding occurs during the southbound migration (Guberti *et al.*, 2007). Birds that migrate from Northern and Eastern Europe congregate in Central Europe for several weeks before commencing the final stage of their southbound migration. During this period, birds may experience multiple infections with different subtypes, and would most likely develop an immunity to the most prevalent strains. An increasing level of population immunity would most probably lead to a progressive decrease in the levels of infection. For this reason it is possible that the levels of virus shedding in the majority of birds that reach Italy are

influenced by the time interval between infection and sampling, by the distance covered and by the level of population immunity to prevalent subtypes. The perpetuation of the endemic cycle of AI under these conditions can probably only be guaranteed by the great numbers of birds present at one time in the wetlands (Delogu *et al.*, 2003).

A noteworthy finding of this investigation is the difference in the isolation rates of H5 and H7 viruses over the two year period. During 2004-2005 there appeared to be a greater isolation rate of H7 viruses (8/11) and no H5 viruses were detected. In contrast during 2005-2006 the prevalence of H5 (3/37) increased and the prevalence of the isolates typed as H7 subtype (13/37) decreased. The reason for this remains to be clarified.

The present investigation also includes the first isolation of an AIV from a wader (common snipe) in Italy. These birds are thought to play a significant role in AI epidemiology in North America (Stallknecht & Shane, 1988), while in Europe their role is still to be fully understood. Another unusual finding is the isolation of an AI virus from a great flamingo. These African birds, have started colonising selected areas of Italian wetlands since the 1990's (Sardinia, Apulia, Emilia-Romagna). Recently they have also colonised areas in Veneto and Sicily. Although their migratory behaviour is still under study, they are now considered semi-resident birds. Their presence in areas which are colonised by infected waterfowl may have unpredictable eco-epidemiological implications. In this study we collected 678 samples from charadriiform birds (waders, terns and gulls) and of these only one yielded a viable isolate. This rate of isolation success is much lower (less than 0.15% of all collected samples) than in the Order of *Anseriformes* (approximately 1.5%).

The reason behind the low isolation rate from samples collected from *Charadriiformes* remains unclear. However, RRT-PCR positive samples collected from *Charadriiformes* were proportionally less than those collected from *Anseriformes*.

The findings in backyard flocks indicate that this type of farming is at high risk for AI introduction. This is primarily due to the fact that these holdings are mostly free-range. The

availability of food attracts wild birds and results in intermingling and in the deposition of droppings. All these conditions favour the introduction of AI into domesticated birds.

The isolation of two virtually identical H7N7 isolates (Figure 3) from a wild mallard and domestic waterfowl confirms this thesis. The risk of introduction of AIVs into free-range farms becomes very high during the period in which the highest number of natural reservoirs is present in the wetlands (autumn and winter). This is supported by the high isolation rate of AI viruses (74%) in backyard flocks during the colder months.

Penetration of AIVs into intensively farmed poultry may occur following amplification of infection in the domestic reservoir. It is also possible that through trade or movement of backyard birds or of contaminated organic material and subsequent infection may spread significantly, thus representing an increased risk for intensively reared birds.

Regardless of the size of the flock, the role of free-range birds in acting as index cases of AIV introduction from the wild host has been highlighted in recent HPAI outbreaks occurring in Europe (Romania, Sweden, Germany, Denmark) (Euro Surveillance, 2006a; Euro Surveillance, 2006b). Given the increased public perception of AI infections and the animal and human health issues, the issue of protecting free range birds from infection should be addressed in a harmonised manner across the European Union.

The wealth of isolates that are now available across Europe and in other parts of the world, due to increased surveillance efforts, should be perceived and managed as a unique opportunity to increase our knowledge on AI and thus contribute to improve animal and public health. The degree of reassortment that is occurring in the wild bird population and the exchange of genes between viruses originating from different hosts should be investigated, along with the genetic variations that occur as AIVs infect new ecosystems and hosts. It is imperative that information collected in national efforts should be seen as part of a pan-European scenario and collated in order to maximise the practical outcome of such programmes.

Acknowledgements

The authors gratefully acknowledge all the staff of the Virology and Research and Development labs for their invaluable technical assistance and Dr. Mauro Delogu of the University of Bologna and Dr. Giuseppe Barigazzi of the Department of Parma of IZSLER who supplied the Italian LPAI H5N1.

This research was funded in part by the Italian Minister of Health (RC IZSVE 003/2001) and the Veneto Region (DGR 3398/05).

References

- Alexander, D.J. & D. Spackman, D. (1981). Characterisation of influenza A viruses isolated from turkeys in England during March-May 1979. *Avian Pathology*, 10, 281-293.
- Campitelli, L., Mogavero, E., De Marco, M.A., Delogu, M., Puzelli, S., Frezza, F., Facchini, M., Chiapponi, C., Foni, E., Cordioli, P., Webby, R., Barigazzi, G., Webster, R.G. & Donatelli, I. (2004). Interspecies transmission of an H7N3 influenza virus from wild birds to intensively reared domestic poultry in Italy. *Virology*, 323, 24-26.
- Capua, I. & Marangon, S. (2000). The avian influenza epidemic in Italy, 1999-2000: a review. *Avian Pathology*, 29, 289-294.
- Capua, I., Marangon, S., & Cancellotti, F.M. (2003). The 1999-2000 avian influenza (H7N1) epidemic in Italy. *Veterinary Research Communication*, 27, 123-127.
- Capua, I., Marangon, S., Selli, L., Alexander, D.J., Swayne, D.E., Dalla Pozza, M., Parenti, E. & Cancellotti, F.M. (1999). Outbreaks of highly pathogenic avian influenza (H5N2) in Italy during October 1997 to January 1998. *Avian Pathology*, 28, 455-460.

- Cattoli, G., Drago, A., Maniero, S., Toffan, A., Bertoli, E., Fassina, S., Terregino, C., Robbi, C., Vicenzoni, G., & Capua, I. (2004). Comparison of three rapid detection systems for type A influenza virus on tracheal swabs of experimentally and naturally infected birds. *Avian Pathology*, 33, 432-437.
- CEC (1992). Council Directive 92/40/EEC of 19 May 1992 introducing Community measures for the control of avian influenza. *Official Journal of the European Commission*, L167, 1-15.
- Chelini, A. (1984). *Le anatre selvatiche*. Firenze: Editoriale Olimpia.
- De Marco, M.A., Foni, E., Campitelli, L., Delogu, M., Raffini, E., Chiapponi, C., Barigazzi, G., Cordioli, P., Di Trani, L. & Donatelli, I. (2005). Influenza virus circulation in wild aquatic birds in Italy during H5N2 and H7N1 poultry epidemic periods (1998 to 2000). *Avian Pathology*, 34(6), 480-485.
- Delogu, M., De Marco, M.A., Donatelli, I., Campitelli, L. & Catelli, E. (2003). Ecological aspects of influenza A virus circulation in wild birds of the western palearctic. *Veterinary Research Communications*, 27, 101-106.
- Euro Surveillance. (2006a). Avian influenza H5N1 detected in German poultry and a United Kingdom wild bird. *Euro Surveillance*, Apr 6;11(4), E060406.1. Hypertext Source: <http://www.eurosurveillance.org/ew/2006/060406.asp>
- Euro Surveillance. (2006b). Avian influenza H5N1 outbreaks in Romanian and Danish poultry, and large H5N1 cluster in an Indonesian family. *Euro Surveillance*, May 25;11(5), E060525.1. Hypertext Source: <http://www.eurosurveillance.org/ew/2006/060525.asp>
- Fouchier, R.A.M., Olsen, B., Bestebroer, T.M., Herfst, S., van der Kemp, L., Rimmelzwaan, G.F., & Osterhaus, A.D.M.E. (2003). Influenza A virus surveillance in wild birds in Northern Europe in 1999 and 2000. *Avian Diseases*, 47, 857-860.
- Gilbert, M., Xiao, X., Domenech, J., Lubroth, J., Martin, V. & Slingenbergh, J. (2006). Anatidae migration in the Western Palearctic and spread of highly pathogenic avian influenza H5N1 virus. *Emerging Infectious Diseases*, 12(11), 1650-1656.

- Guberti, V., Scremin, M., Busani, L., Bonfanti, L. & Terregino, C. (2007). A simulation model for the LPAI viruses in dabbling ducks in Europe. *Avian Diseases*. (in press).
- Hillis, D.M. & Bull, J.J. (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology*, 42(2), 182-192.
- Hinshaw, V.S., Webster, R.G. & Turner, B. (1980). The perpetuation of Orthomyxoviruses and paramyxoviruses in Canadian waterfowl. *Canadian Journal of Microbiology*, 26, 622-629.
- Hoffman, E., Stech, J., Guan, Y., Webster, R.G., & Perez, D.R. (2001). Universal primer set for the full-length amplification of all influenza A viruses. *Archives of Virology*, 146, 2275-2289.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA 3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, 5(2), 150-163.
- Munster, V., Wallensten, A., Olsen, B., Rimmelzwaan, G.F., Osterhaus, A.D.M.E., & Fouchier, R.A.M. (2005). Influenza A virus surveillance in wild birds. *Avian influenza: Prevention and control*. Schrijver RS, Koch G (Eds), Softcover ISBN 1-4020-3440-7, Chapter 4, 25-30.
- Munster, V.J., Veen, J., Olsen, B., Vogel, R., Osterhaus, A.D.M.E., & Fouchier, R.A.M. (2006). Towards improved influenza A virus surveillance in migrating birds. *Vaccine*, 24(44-46), 6729-6733.
- Nei, M., & Kumar, S. (2000) *Molecular evolution and phylogenetics*. Oxford University Press, Inc. New York, (pp37-38).
- Okazaki, K., Takada, A., Ito, T., Imai, M., Takakuwa, H., Hatta, M., Ozaki, H., Tanizaki, T., Nagano, T., Ninomiya, A., Demenev, V.A., Tyaptirganov, M.M., Karatayeva, T.D., Yamnikova, S.S., Lvov, D.K. & Kida, H. (2000). Precursor genes of future pandemic influenza viruses are perpetuated in ducks nesting in Siberia. *Archives of virology*, 145(5), 885-893.
- Olsen, B., Munster, V.J., Wallensten, A., Waldenstrom, J., Osterhaus, A.D., & Fouchier, R.A. (2006). Global patterns of influenza A virus in wild birds. *Science*, 312(5772), 384-388.

- Spackman, E., Senne, D.A., Myers, T.J., Bulaga, L.L., Garber, L.P., Perdue, M.L., Lohman, K., Daum, L.T. & Suarez, D.L. (2002). Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *Journal of Clinical Microbiology*, 40, 3256-3260.
- Stallknecht, D.E. & Shane, M. (1988). Host range of avian influenza virus in free-living birds. *Veterinary Research Communications*, 12, 125-141.
- Terregino, C., Cattoli, G., Guberti, V., De Nardi, R., Scremin, M., Beato, M.S. & Capua, I. (2005). Isolation of Influenza A viruses belonging to the H7N7 and H7N4 subtype from wild and domestic waterfowl in Italy. *The Veterinary Record*, 26, 292.
- Webster, R.G. & Hulse, D.J. (2004). Microbial adaptation and change: avian influenza. *Revue Scientifique et Technique*, 23(2), 453-465.

Table 1. Main wild bird species (≥ 10 subjects) tested during the study period. Results of RRRT-PCR analyses and virus isolation

Species	N. of samples collected	N. of AIV positives by Type A	N. of AIV positives by RRRT-PCR (%)		Isolates	N. of isolates (%)
			H5	H7		
Mallard (<i>Anas platyrhynchos</i>)	1,558	124 (8.0)	6 (0.4)	21 (1.3)	5 x H1N1, H3N8, 2 x H4N6, 12 x H7N7, H7N4, H5N1, 2 x H10N7, H10N8, 2 x H9N2, H11N9	28 (1.8)
Northern Shoveler (<i>Anas clypeata</i>)	173	18 (10.4)	1 (0.6)	3 (1.7)	4 x H7N7, H10N7	5 (2.9)
Great Cormorant (<i>Phalacrocorax carbo</i>)	35	2 (5.7)	0 (0.0)	0 (0.0)	0	0 (0.0)
Common Teal (<i>Anas crecca</i>)	559	83 (14.8)	4 (0.7)	3 (0.5)	3 x H7N7, H5N3, H5N2, H1N1, H1N3, H10N7, H3N8, H4N6	10 (1.6)
Northern Pintail (<i>Anas acuta</i>)	142	4 (2.8)	0 (0.0)	1 (0.7)	H10N4, H1N1	2 (1.4)
Greater Flamingo (<i>Phoenicopterus roseus</i>)	75	19 (25.3)	0 (0.0)	0 (0.0)	H6N2	1 (1.3)
Eurasian Wigeon (<i>Anas penelope</i>)	503	36 (7.2)	0 (0.0)	0 (0.0)	H6N2, H3N8	2 (0.4)
Eurasian Coot (<i>Fulica atra</i>)	103	2 (1.9)	0 (0.0)	1 (1.0)	0	0 (0.0)
Black-headed Gull (<i>Larus ridibundus</i>)	96	0 (0.0)	0 (0.0)	0 (0.0)	0	0 (0.0)
Dunlin (<i>Calidris alpina</i>)	172	9 (5.2)	0 (0.0)	0 (0.0)	0	0 (0.0)
Common Pochard (<i>Aythya ferina</i>)	130	3 (2.3)	0 (0.0)	0 (0.0)	0	0 (0.0)
Sandwich Tern (<i>Sterna sandvicensis</i>)	150	11 (7.3)	0 (0.0)	0 (0.0)	0	0 (0.0)
Common Tern (<i>Sterna hirundo</i>)	134	4 (3.0)	0 (0.0)	0 (0.0)	0	0 (0.0)
Ruff (<i>Philomachus pugnax</i>)	16	6 (3.0)	1 (6.3)	0 (0.0)	0	0 (0.0)
Gadwall (<i>Anas strepera</i>)	58	2 (3.4)	0 (0.0)	0 (0.0)	0	0 (0.0)
Tufted Duck (<i>Aythya fuligula</i>)	12	0 (0.0)	0 (0.0)	0 (0.0)	0	0 (0.0)
Black Tern (<i>Chlidonias niger</i>)	18	1 (5.5)	0 (0.0)	0 (0.0)	0	0 (0.0)
Eurasian Woodcock (<i>Scolopax rusticola</i>)	16	0 (0.0)	0 (0.0)	0 (0.0)	0	0 (0.0)
Little Tern (<i>Sterna albifrons</i>)	17	0 (0.0)	0 (0.0)	0 (0.0)	0	0 (0.0)
Mediterranean Gull (<i>Larus melanocephalus</i>)	39	1 (2.6)	0 (0.0)	0 (0.0)	0	0 (0.0)
Wood Pigeon (<i>Columba palumbus</i>)	23	0 (0.0)	0 (0.0)	0 (0.0)	0	0 (0.0)
Common Snipe (<i>Gallinago gallinago</i>)	10	1 (10)	0 (0.0)	0 (0.0)	H10N7	1 (10.0)

Table 2. Avian influenza isolates in Italy in 2004-2005 in backyard flocks

Year	Region	Species	Subtype
2004	Emilia Romagna	Pekin duck (<i>Anas platyrhynchos</i> f. dom.)	2xH1N1, 1xH4N6
		Goose (<i>Anser anser</i> f. dom.)	1xH3N8, 1xH4N2, 1xH11N9
	Lombardia	Mallard (<i>Anas platyrhynchos</i>) and Pekin duck (<i>Anas platyrhynchos</i> f. dom.)	1xH1N2 ^a
		Mallard (<i>Anas platyrhynchos</i>) and Pekin duck (<i>Anas platyrhynchos</i> f. dom.)	1xH9N8 ^a
		Mallard (<i>Anas platyrhynchos</i>) and Pekin duck (<i>Anas platyrhynchos</i> f. dom.)	1xH1N1 ^a
		Mallard (<i>Anas platyrhynchos</i>)	1xH5N3 LPAI, 1xH10N4
	Veneto	Pekin duck (<i>Anas platyrhynchos</i> f. dom.) and goose (<i>Anser anser</i> f. dom.)	1xH10N4 ^a
		Muscovy duck (<i>Cairina moschata</i>)	1xH7N7 LPAI
		Goose (<i>Anser anser</i> f. dom.)	1xH7N7 LPAI
	Emilia Romagna	Pekin duck (<i>Anas platyrhynchos</i> f. dom.)	1xH1N1, 1xH10N7, 1xH11N9, 1xH4N6
		Mallard (<i>Anas platyrhynchos</i>)	1xH4N6
2005	Lombardia	Muscovy duck (<i>Cairina moschata</i>) and goose (<i>Anser anser</i> f. dom.)	1xH2N2 ^a
		Mallard (<i>Anas platyrhynchos</i>)	1xH11N9, 1xH1N1
		Mallard (<i>Anas platyrhynchos</i>) and goose (<i>Anser anser</i> f. dom.)	1xH11N9 ^a
	Veneto	Mallard (<i>Anas platyrhynchos</i>) and Pekin duck (<i>Anas platyrhynchos</i> f. dom.)	1xH11N2 ^a
		Guinea fowl (<i>Numida meleagris</i>)	1xH9N2
		Pekin duck (<i>Anas platyrhynchos</i> f. dom.)	2x H11N9

^a Viruses isolated from pool of faeces of the two species.

Table 3. AI isolates from wild birds in each region in the winter seasons 2004/05 and 2005/06

Season	Region	Species	Subtype
Winter season 2004/05	Emilia Romagna	Mallard (<i>Anas platyrhynchos</i>)	1xH7N7 LPAI
		Greater Flamingo (<i>Phoenicopterus roseus</i>)	1xH6N2
		Northern Pintail (<i>Anas acuta</i>)	1xH10N4
	Veneto	Mallard (<i>Anas platyrhynchos</i>)	1xH1N1, 1xH4N6, 2xH7N7 LPAI, 1xH7N4 LPAI
		Common Teal (<i>Anas crecca</i>)	1xH7N7 LPAI
	Lombardia	Common Teal (<i>Anas crecca</i>)	1xH7N7 LPAI
		Mallard (<i>Anas platyrhynchos</i>)	1xH7N7 LPAI
	Emilia Romagna	Mallard (<i>Anas platyrhynchos</i>)	1xH5N1 LPAI
	Veneto	Mallard (<i>Anas platyrhynchos</i>)	1xH3N8, 1xH10N7, 1xH10N8, 8xH7N7 LPAI, 3xH1N1, 2xH9N2
		Common Snipe (<i>Gallinago gallinago</i>)	1xH10N7
Winter season 2005/06	Lombardia	Northern Shoveler (<i>Anas clypeata</i>)	4xH7N7 LPAI, 1xH10N7
		Common Teal (<i>Anas crecca</i>)	1xH5N3, 1xH1N1, 1xH1N3, 1xH7N7, 1xH10N7, 1xH3N8, 1xH4N6
		Eurasian Wigeon (<i>Anas penelope</i>)	1xH6N2, 1xH3N8
		Northern Pintail (<i>Anas acuta</i>)	1xH1N1
		Mallard (<i>Anas platyrhynchos</i>)	1xH1N1, 1xH10N7, 1xH4N6, 1xH11N9
		Common Teal (<i>Anas crecca</i>)	1xH5N2 LPAI

Figure 1: *Densely populated poultry area (DPPA) in Northern Italy (1a); Collection sites from migratory wild birds (1b)*

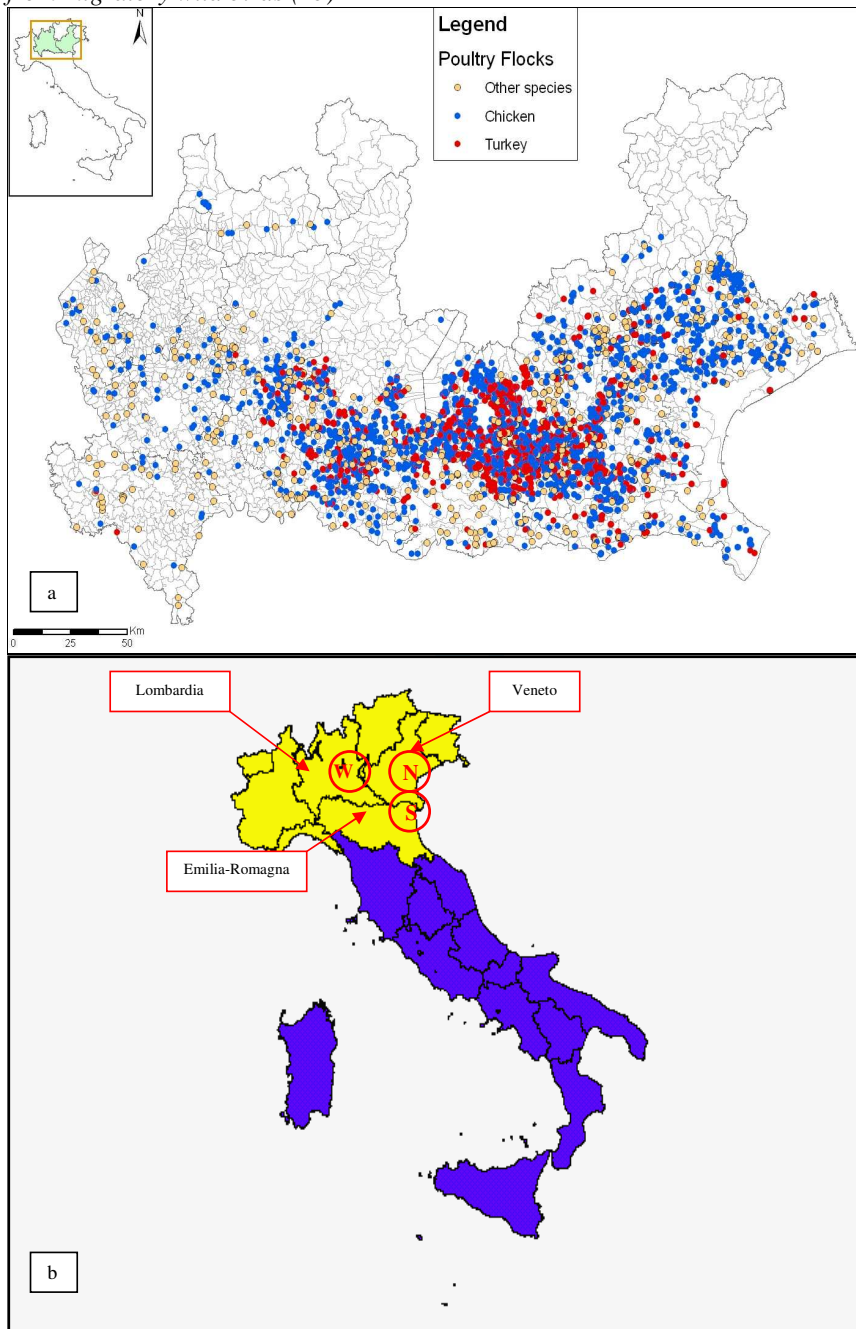
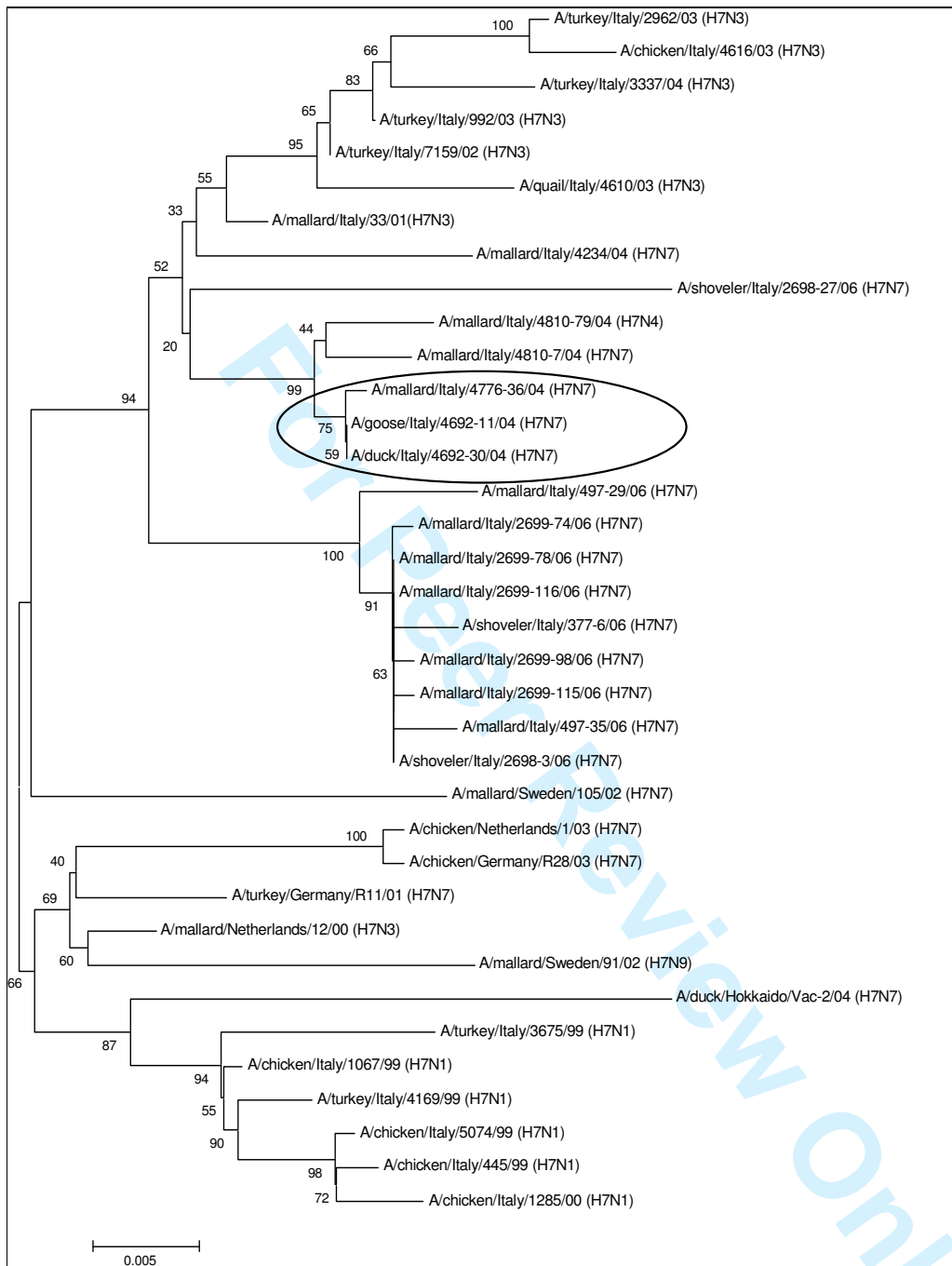


Figure 2: European flyways of mallard (2 a) and common teal (2 b) (Chelini, 1984)



Figure 3: Phylogenetic analysis of the genome segment encoding for the H7 strains isolated from domestic and wild birds



Note: The strains in the oval (A/duck/Italy/4692-30/04, A/goose/Italy/4692-11/04 and A/mallard/Italy/4776-36/04) isolated respectively from domestic ducks and geese and from wild mallard in the 2004/2005 winter season cluster together.

Figure 4: *Phylogenetic analysis of the genome segment encoding for the H5 strains isolated from domestic and wild birds*

