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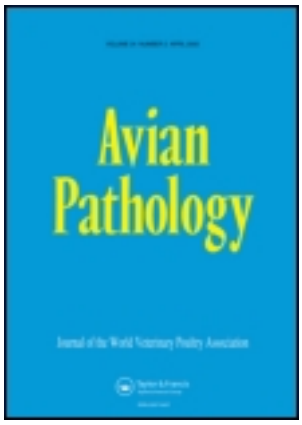

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Multiyear surveillance of influenza A virus in wild birds in Portugal

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Multiyear surveillance of influenza A virus in wild birds in Portugal

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This report presents the results of a multiyear (2005 to 2009) study of avian influenza virus (AIV) occurrence in wild birds in Portugal. A total of 5691 samples from wild birds belonging to 13 different orders were examined. Ninety-three samples tested positive for AIV by matrix reverse transcriptase-polymerase chain reaction, giving a total prevalence of 1.63%. Twenty-one viruses were successfully cultured in embryonated chicken eggs, which represent a rate of viral infectivity of 22.6% in the samples. Nine subtypes of haemagglutinin (H1, H3 to H7, H9 to H11) and eight subtypes of neuraminidase (N1 to N4, N6 to N9) were identified in 20 different combinations. The most prevalent subtypes of haemagglutinin detected were H5, H1 and H4, while for neuraminidase subtypes N2 and N6 were the most common. The subtype combinations H4N6 and H1N1 were predominant (15.1%). All H5 and H7 viruses detected in the present study were low pathogenic for poultry as determined by the sequence of amino acids at the cleavage site of haemagglutinin. The full-length nucleotide sequences of five H5, one H7 and five N3 genes were analysed phylogenetically. The Bayesian analysis revealed that all but one of the strains analysed were closely related to isolates detected in the same period in North and Central European countries. Three H5N3 isolates, all from 2007, formed a separate cluster in both H5 and N3 phylogenetic trees. This study provides evidence that various subtypes of AIV, including subtypes H5 and H7, circulate in Portugal, which may pose a risk to industrial poultry.

Introduction

The influenza A virus, and most of its haemagglutinin (HA) and neuraminidase (NA) subtype combinations, is commonly present in wild bird species of wetlands and aquatic environments, with most infections thought to be inapparent (Webster *et al.*, 1992; Swayne & Suarez, 2000). Clinically, avian influenza viruses (AIVs) can be classified into two main pathotypes (Capua & Alexander, 2004): the highly pathogenic avian influenza (HPAI) viruses that cause a systemic disease in which mortality may approach 100% in fowl; and low-pathogenic avian influenza (LPAI) viruses that cause a much milder disease in susceptible poultry. HPAI is caused only by certain strains of H5 and H7 viruses.

The segmented nature of the AIV genome enables viral evolution by genetic reassortment. A recent study indicates that genetic sublineages do not persist, but frequently reassort with other viruses (Munster *et al.*, 2007). Gaining information on the actual frequency of reassortment in the wild bird population and the impact of these events on LPAI virus evolution is of relevant interest.

AIVs have diversified into two separate avian lineages, North American and European, probably as a consequence of long-term ecological and geographical separation of the hosts (Krauss *et al.*, 2004; Olsen *et al.*, 2006). Several multi-year studies have been made in countries of Northern Europe in wild birds (Fouchier *et al.*, 2003; Munster *et al.*, 2007; Wallensten *et al.*, 2007) but to our knowledge no such study has so far been conducted in Portugal. Situated in the east Atlantic migratory flyway, the country is visited by many species that migrate annually between Africa and the Iberian Peninsula, mainly from March to July, which corresponds with spring migration and the breeding season for birds. On the other hand, wintering migratory birds, especially waterfowl, come predominantly from the northern regions of Europe. These two migratory populations mingle with resident free-living birds, which may favour the introduction of new AIV in the country. In the frame of avian influenza preparedness, a surveillance programme was carried out in wild birds aiming to assess the risk of AIV introduction, either of low (LPAI) or high (HPAI) pathogenicity to domestic poultry. The present study reports the epidemiological and virological findings of AIV occurrence in wild birds in Portugal from January 2005 to December 2009.

Materials and Methods

Collection of samples. The majority of samples originated from migratory or resident wild birds and were collected in bird sanctuaries

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located in the vicinity of poultry farms. The birds were caught throughout the year. A total of 5691 samples consisting of swabs collected from the oropharynx and cloaca of wild birds belonging to 13 different orders were analysed during the 5-year study (Table 1). The swabs were transported dried and kept at 5 to 8°C until tested.

Avian influenza virus screening. The contents of the oropharyngeal and cloacal swabs was eluted and pooled into vials containing 2 ml cold phosphate-buffered saline with antibiotics (2000 u/ml penicillin, 2 mg/ml streptomycin and 50 µg/ml gentamycin) and submitted to RNA extraction on a nucleic acid extraction workstation BioSprint 96, following the manufacturer's protocol (Qiagen, Hilden, Germany). The samples were firstly screened for AIV by matrix (M) gene reverse transcriptase-polymerase chain reaction (rRT-PCR) using the method described by Spackman *et al.* (2002) and the Qiagen One-step rRT-PCR kit in 25 µl final volume. Positive and negative controls were included in each assay.

Virus subtyping. Matrix rRT-PCR positive samples were further screened for subtypes H5 and H7, using rRT-PCR protocols recommended by the EU Community Reference Laboratory (Slomka *et al.*, 2007b, 2009). To determine all other AIV subtypes, the RNA obtained from swabs or tissues was used to partially amplify the HA and NA genes by conventional RT-PCR with universal primers described by Hoffmann *et al.* (2001) and primers designed in our laboratory (NA-F, CAC CAT GGA TCC AAA TCA GAA GAT; N7-F, CAC CAT GGA TCC AAA TC AGA AAC TAT TTG; NeurUniR, CCD AWY CTD AYN SCR TTG TCY TT), respectively. The RT-PCR products were analysed by 2% (w/v) agarose gel electrophoresis and the amplicons were purified after being excised from the gel using the Qiagen QIAquick gel extraction kit as described by the manufacturer. Nucleotide sequencing was carried out with the BigDye DNA sequencing kit (Applied Biosystems, Foster City, USA) in the Applied Biosystems 3130 genetic analyser.

Virus pathotyping. The RNA obtained from swabs or tissues was used to accomplish the pathotyping of H5 and H7 viruses. The genomic region encoding the HA protein cleavage site was amplified with primer pairs recommended by the EU Community Reference Laboratory, Kha1/Kha3 for H5 and GK7.3/ GK7.4 for H7 (Slomka *et al.*, 2007a) and the nucleotide sequences of the purified PCR products were determined as described above.

Virus culture. Virus isolation in specific-pathogen-free 11-day-old embryonated chicken eggs was also attempted with all matrix rRT-PCR-positive samples. Samples testing negative after the first passage were passaged twice more to confirm the absence of AIV. Haemagglutinating-positive allantoic fluids were tested against reference sera to H5, H7 and Newcastle disease virus (as described in Chapter V of the *EU Diagnostic Manual*) and for the M, H5 and H7 genes by rRT-PCR as described above.

Table 1. *Total number of wild birds screened for influenza A virus (2005 to 2009).*

Bird order	2005	2006	2007	2008	2009	Total
Accipitriformes	54	83	14	18	33	202
Anseriformes	361	1479	637	787	297	3561
Charadriiformes	6	58	70	12	181	327
Ciconiiformes	70	208	37	17	14	346
Falconiformes	27	28	3	6	12	76
Galliformes	6	0	0	1	0	7
Gruiformes	6	57	0	12	30	105
Passeriformes	32	170	7	66	11	286
Pelecaniformes	26	135	26	4	3	194
Phoenicopteriformes	23	159	3	2	1	188
Podicipediformes	2	6	1	0	0	9
Procellariiformes	5	17	35	90	109	256
Strigiformes	29	63	10	8	24	134
Total	647	2463	843	1023	715	5691

Phylogenetic analysis. The complete nucleotide sequences of five H5, one H7 and five N3 genes were determined and analysed phylogenetically. The viruses included in the study were selected after BLAST analysis and comprised not only European strains showing high homology to sequenced viruses but also viruses from other parts of the world. Only complete sequences were used in the analysis. The phylogenetic trees were obtained with a Bayesian inference of phylogeny throughout the MrBayes v3.1.2 software, using the GTR model (nst = 6) with gamma-shaped rate variation with a proportion of invariable sites (rates = invgamma). The analysis used ngen = 10^6, nchains = 4 and samplefreq = 10.

Accession numbers. The GenBank accession numbers HM849026 to HM849030 and HM849003 represent the H5 and H7 genes of 28003/H5N3, 28006/H5N3, 29497-10/H5N2, 37369/H5N3, 4228/H5N3, and 13771/H7N3, respectively. The N3 genes of 13771/H7N3, 28003/H5N3, 28006/H5N3, 37369/H5N3 and 4228/H5N3 were given, respectively, accession numbers HM849005 and HM849031 to HM849034.

Results and Discussion

AIV detection and virus isolation. The total prevalence of influenza A virus in the birds sampled during the 5-year period was 1.63%. The large proportion (88.2%) of the 93 AIV-matrix gene rRT-PCR-positive samples was from mallards, and only 11 samples came from other species of ducks, and from seagulls, storks, partridges and flamingos. Samples taken from live birds made up 70% of the total and 76.3% of the rRT-PCR-positive samples, thus demonstrating the importance of monitoring live birds in the success of avian influenza surveillance. The main places of the sampling as well as the districts where the positive birds were found are shown in Figure 1. Figure 2 represents the temporal distribution of sampling and rRT-PCR-positive samples.

Twenty-one viruses were successfully cultured in embryonated chicken eggs, which represent a rate of viral infectivity (number of viruses isolated/number of rRT-PCR-positive samples) of 22.6% in the samples (Table 2). The swabs were transported dried, which may have affected the success of AIV isolation. However, the low rate of virus isolation recorded in present study is consistent with that found in other studies (Munster *et al.*, 2007; Dusek *et al.*, 2009). Usually, rRT-PCR is more sensitive than virus isolation, enabling detection of genome fragments of non-infective viruses.

Virus subtyping. In this study, nine subtypes of HA (H1, H3 to H7, H9 to H11) and eight subtypes of NA (N1 to N4, N6 to N9) were identified in 20 different combinations (Figure 3). The most prevalent subtypes of HA detected were H5 (23.7%), H1 and H4 (21.5%). The high prevalence of H5 subtype is unusual and different to that found in other studies in Europe and North America. However, this fact can be explained by the increased sampling of a population of mallards semi-resident in a natural reserve, after the detection of H5N2. As in other studies (Suss *et al.*, 1994; Krauss *et al.*, 2004; Wallensten *et al.*, 2007), H4 and H1 also dominated, followed by H9 and H6, while subtypes H2, H8 and H12 to H16 were never detected in this study. Concerning NA, the most

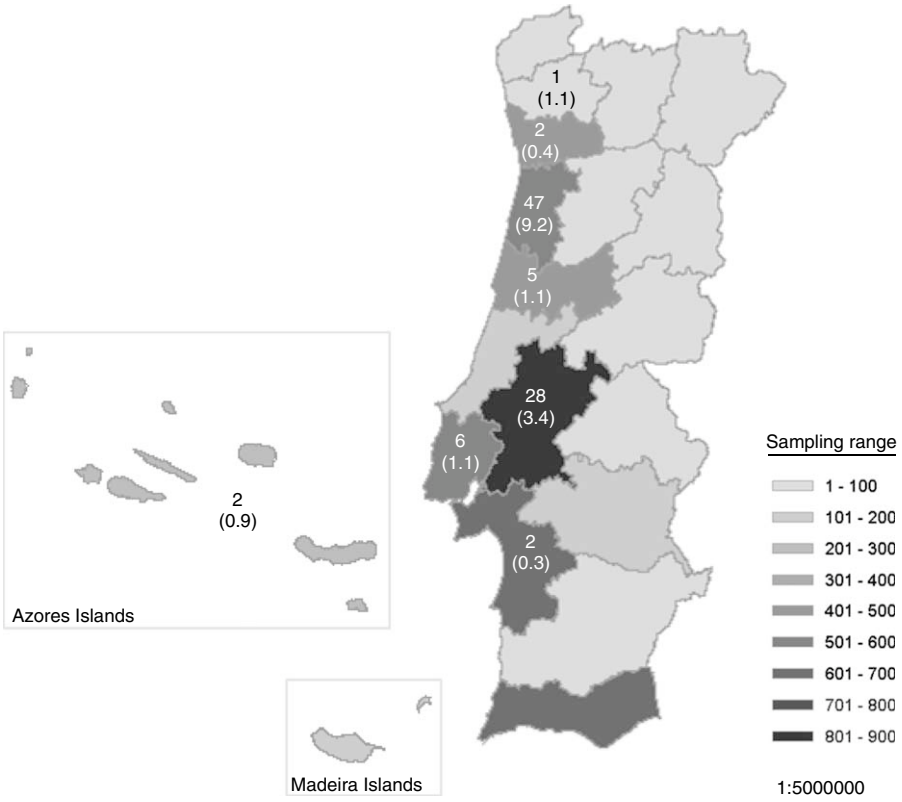

Figure 1. *Map of Portugal presenting the distribution of the AIV sampling by district. The number of positive samples, with percentage in brackets, is indicated.*

prevalent subtype was N2 (32.3%), followed by N6 (17.2%). With the exception of N5, all other known subtypes of NA were found. The subtype combinations H4N6 and H1N1 (15.1%) were the most common, similar to rates previously reported in Sweden and Germany (Suss *et al.*, 1994; Wallensten *et al.*, 2007). Our study also shows that H5N3 and H5N2 combinations were frequently detected in Portugal, with prevalence of 10.8% and 7.5%, respectively.

Virus pathotyping. The 22 H5 and four H7 AIV identified in this study were found to be of low pathogenicity for poultry since they lack basic amino acid residues at the cleavage site of HA. The pathogenicity of four isolates was also assessed by plaque-forming assays in Madin Darby Canine Kidney (MDCK) cells in the presence and absence of trypsin. The isolates tested, one H7N3 and three H5N3, required trypsin for plaque formation, thus corroborating the molecular prediction of low pathogenicity.

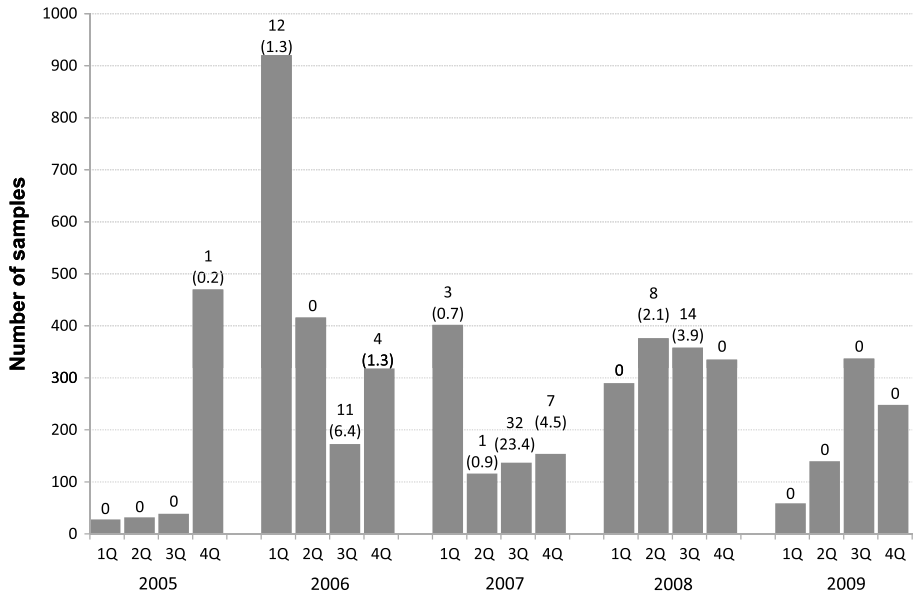

Figure 2. *Temporal distribution of samples. The bars represent the sampling distribution. The number of AIV positive birds, with percentage in brackets, is indicated.*

Table 2. *AIV prevalence and subtypes found in wild birds (2005 to 2009).*

Common name (scientific name)	Number tested	Number of positives (%)	Viable virus (%)	Subtype (number of positives)
Mallard (*Anas platyrhynchos*)	1495	82 (5.5)	20 (24.4)	H1N1 (14), H1N2 (3), H1N6 (1), H1N? (1), H3N2 (1), H3N3 (1), H3N8 (1), H4N2 (5), H4N6 (14) H4N? (1), H5N2 (6), H5N3 (9), H5N6 (1), H5N? (3), H6N2 (1), H6N7 (1), H6N? (1); H7N3 (1); H9N2 (6), H9N4 (1), H9N? (1); H10N2 (2), H11N9 (5), H?N2 (2)
Common teal (*Anas crecca*)	204	3 (1.5)	0	H1N2 (1), H7N7 (1), H9N2 (1)
Tufted duck (*Aythya fuligula*)	24	1(4.2)	1 (100)	H7N3 (1)
Seagull (*Larus* sp.)	522	3 (0.6)	0	H6N2 (1), H6N3 (1), H6N? (1)
Flamingo (*Phoenicopterus* sp.)	194	1 (0.5)	0	H5N2 (1)
Partridge (*Perdix perdix*)	227	2 (0.9)	0	H5N3 (1); H5N? (1)
Stork (*Ciconia ciconia*)	178	1 (0.6)	0	H7N? (1)

Nucleotide sequence analysis. The molecular analysis of non-structural 1 (NS1) proteins and of the stalk region of neuraminidase of these strains revealed full-length proteins, typical of viruses from wild birds (Matrosovich *et al.*, 1999). In addition, all viruses studied possess Asp92 and the ESEV amino acid motif at the C-terminal of NS1 protein, typical of AIVs (Dundon *et al.*, 2006; Soubies *et al.*, 2010). Analysis of matrix 2 protein (M2) of fully sequenced isolates showed that none of the isolates were resistant to amantidine.

Phylogenetic analysis. Phylogenetic analysis was performed with the complete nucleotide sequence of the H5 gene of five AIVs detected during this study (Figure 4). The analysis revealed that the H5 isolates from this study were closely related to LPAI strains from the North and Central European countries and more distantly related to African, Asian and American viruses. Three strains detected in 2007 (28003/H5N3, 28006/H5N3 and 37369/H5N3) were more closely related to each other and to German isolate A/mallard/Bavaria/35/2006(H5) with 99% nucleotide identity. The other two H5 viruses (4228/H5N3 and 29497-10/H5N2) showed a close relationship with isolates from Sweden and Switzerland (Figure 4a). The identity of the nucleotide sequences of the H5 viruses reported here ranged between 93 and 99%. The 2006 H7 isolate (13771/H7N3) was also analysed, showing greatest nucleotide identity (98 to 99%) with isolates from Hungary, the Netherlands, Sweden and the UK detected between 2005 and 2007 (data not shown).

Five neuraminidases of subtype N3 chosen at random were fully sequenced and analysed phylogenetically. Four of them belonged to H5 strains from ducks and one to a H7 virus isolated from a tufted duck. The nucleotide variation within the N3 sequences of these five viruses ranged between 94 and 99%. Interspersed clusters of European and Asian viruses were observed. American and Oceaniic viruses formed a distinct group, as expected. With the exception of 4228/H5N3, all other strains from this study clustered with European viruses. Three H5N3 viruses (28003/H5N3, 28006/H5N3 and 37369/H5N3) detected in 2007 and with different geographic origins formed a separate branch with a French isolate of 2002. Isolate 13771/H7N3 branched with Swiss and Dutch isolates (Figure 4b).

It was interesting to note that H5N3 strains 28003, 28006 and 37369, all from 2007 but with different geographic origins, clustered together and formed a distinct sub-cluster in both H5 and N3 dendograms. Also of note was strain 4228/H5N3, which may be a reassortant virus since H5 and N3 genes clustered with European and Asian viruses, respectively. The North American and Eurasian virus lineages were clearly evidenced both in H5 and N3 analysis.

The results of this study indicate the active circulation of influenza A viruses in wild birds in Portugal. Amongst all wild birds sampled, mallards harboured the majority of AIV obtained during this multi-year surveillance. The greater susceptibility of mallards to avian influenza infections has also been reported in other studies (Webster *et al.*, 1992; Olsen *et al.*, 2006), this species being considered the main target for sampling in avian influenza surveillance programmes. The isolation of H5 and H7 subtypes and the geographical vicinity between wild-bird sanctuaries and poultry farms represents a risk for industrial poultry of the country.

The present study also provides the genotypic analysis and detailed phylogenetic data of several strains collected during a 5-year period. Compiling sequence data and the identification of new viruses and their relationships will contribute to a better understanding of the evolution of the viruses and also for the establishment of an updated plan for molecular diagnosis of influenza A viruses.

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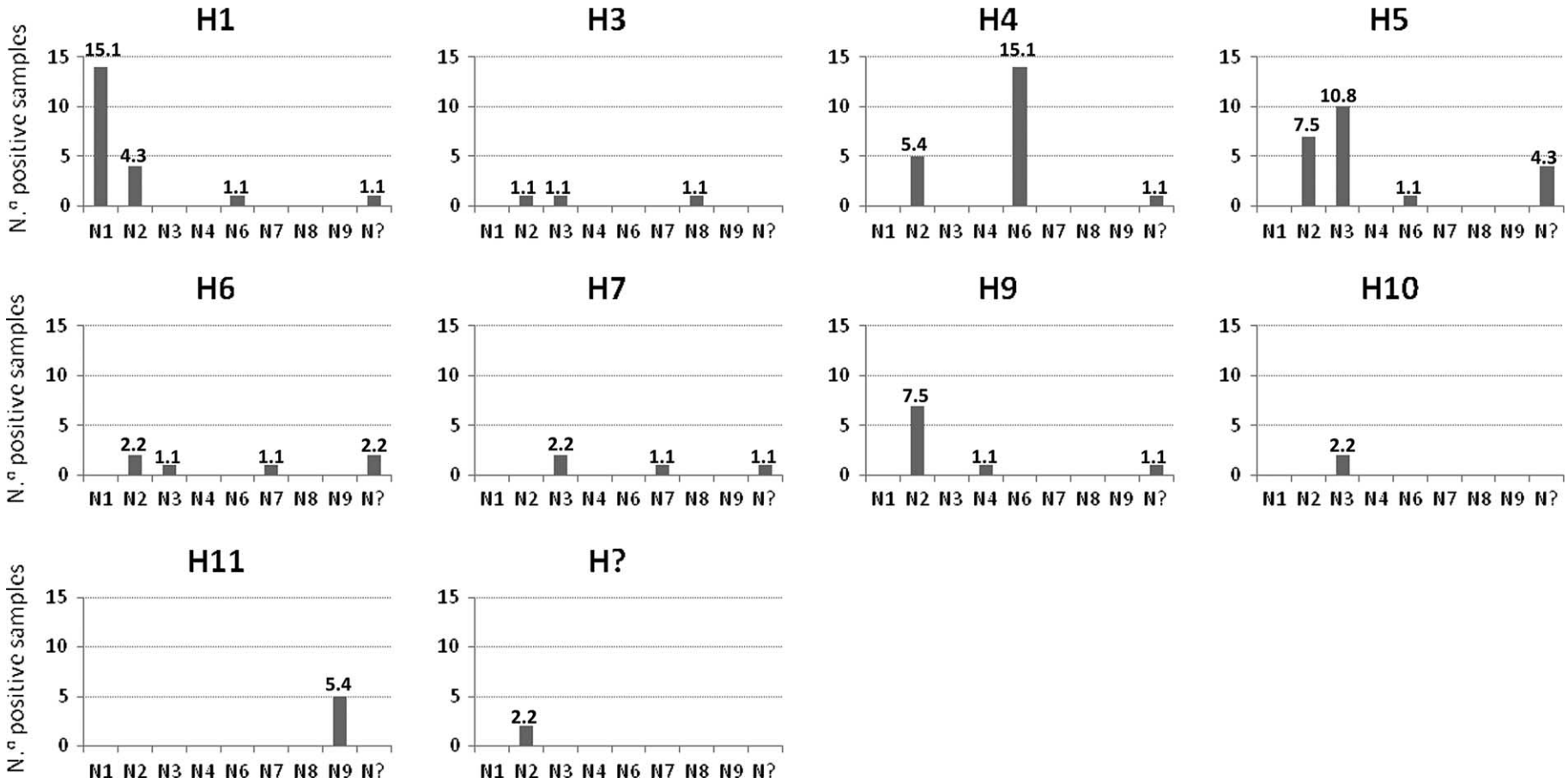

Figure 3. *The HA and NA subtypes of AIV found in wild birds in Portugal during 2005 to 2009. The numbers above the bars in the graphs represent the percentage of positive samples.*

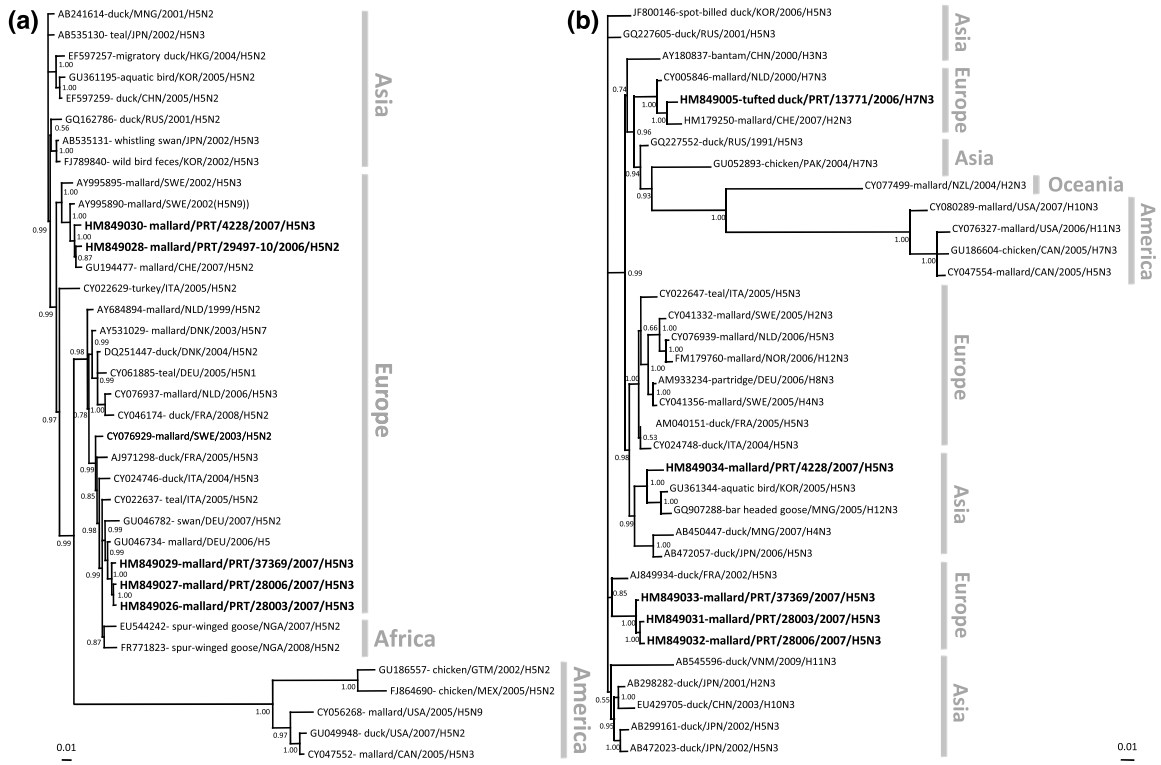

Figure 4. *Bayesian analysis of the complete nucleotide sequences of H5 (4a) and N3 (4b) genes of influenza A virus detected in Portugal in wild ducks during 2005 to 2009. The phylogenetic trees were obtained with a Bayesian inference of phylogeny throughout the MrBayes v3.1.2 software, using the GTR model (nst = 6) with gamma-shaped rate variation with a proportion of invariable sites (rates = invgamma). The analysis used ngen = 10^6, nchains = 4 and samplefreq = 10. The numbers included on each bootstrap represent the Bayesian posterior probability. Viruses reported in this study are presented in bold.*

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