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Authors: Pannwitz, G., Wolf, C., and Harder, T.

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Active Surveillance for Avian Influenza Virus Infection in Wild Birds by Analysis of Avian Fecal Samples from the Environment

G. Pannwitz,[1] C. Wolf,[2] and T. Harder[3,4] [1] District Food and Veterinary Office, Ostvorpommern, Bluthsluster Str. 5b, D-17389, Anklam, Germany; [2] State Office for Agriculture, Food Safety, and Fishery, Mecklenburg and Western Pomerania, Department for Diagnostic Investigation of Epizootics, Thierfelderstraße 18, D-18059, Rostock, Germany; [3] O.I.E. and National Reference Laboratory for Avian Influenza, Institute for Diagnostic Virology, Friedrich-Loeffler Institute, Südufer 10, D-17493 Greifswald-Insel Riems, Germany; [4] Corresponding author (email: timm.harder@fli.bund.de)

ABSTRACT: A total of 1991 environmental samples of fresh avian feces and urine from several aquatic bird species in a coastal area of Northeast Germany were examined for the presence of avian influenza viruses (AIV). By real-time reverse-transcriptase polymerase chain reaction, specific for an M gene of influenza A viruses, none of 659 duck samples and only 11 (0.9%) of 1,268 geese and swan samples tested positive. Two of these were identified as H5N2 viruses of low pathogenicity. Conventional cloacal and oropharyngeal swab samples (n=1,402) collected in an adjacent coastal region in Northeast Germany from comparable species of captured or hunted birds, yielded a similar detection rate (3/901; 0.4%) for AIV-specific RNA in geese and swans, but a higher rate (4/309; 1%) for ducks. No virus isolates were obtained from either set of samples. Collection of environmental avian samples was simple and cost effective and also allowed us to regulate sample sizes over time. A species assignment of these samples was possible, provided that close presampling observation of birds at the sampling sites was secured. Environmental sampling to monitor AIV in wild bird populations may be a valid alternative to the more-invasive and capture-dependent methods based on cloacal sampling.

Key words: Avian influenza, conservation, environment, highly pathogenic avian influenza virus, surveillance.

Wild birds of the orders Anseriformes and Charadriiformes constitute the natural biotic reservoir of avian influenza viruses (AIV) of low pathogenicity (LPAIV). The transmission of LPAIV is governed by fecal–oral infection chains whereby surface water, ice, and benthal sediments may constitute potentially significant abiotic reservoirs (Ito et al., 1995; Zhang et al., 2006; Lang et al., 2007; Stallknecht and Brown, 2007; Webster et al., 2007). LPAIV of subtypes H5 and H7 act as progenitor viruses of highly pathogenic variants (HPAIV) which may arise de novo by mutational events following transmission and circulation of LPAIV of these subtypes in gallinaceous poultry (Alexander, 2007). HPAIV causes devastating disease in poultry and may be retransmitted into wild bird populations (Kilpatrick et al., 2006; Guan et al., 2007; Keawcharoen et al., 2008).

In early 2006, HPAIV H5N1 infections were detected in wild birds on the island of Ruegen, Germany (54°08′N, 13°27′E) and in adjacent regions along the Baltic Sea coast. The island of Ruegen and the surrounding coastal shallows are important staging and wintering areas for a number of migratory, aquatic wild-bird species and, thus, are of international significance with respect to transmissible avian diseases. A significant proportion of the total number of H5N1-infected wild birds found in Europe originated from the Ruegen outbreak which ended in April–May of 2006. This led to intense postoutbreak surveillance of wild birds in this region, including analyses of cloacal and oropharyngeal swabs and of avian fecal samples derived from the environment, in order to detect a possible perpetuation of HPAIV in the absence of clinical cases.

In this paper, we provide evidence that sampling of freshly dropped feces from migratory wild-bird species may yield

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results comparable to those obtained by swab sampling of captured or hunted birds. Thus, analysis of environmental fecal samples may complement standard, active-monitoring efforts for AIV in wild birds.

During a period of 191 days, between 3 September 2006 and 18 March 2007, a total of 1,991 samples of fresh droppings from wild Anseriformes and Charadriiformes birds were collected in Northeast Germany, south of a large inlet of the Baltic Sea, the Greifswalder Bodden. No poultry or poultry manure was present on the ranges sampled. Details of sampling including the establishment of the species origin of droppings, a method that has been described elsewhere (Pannwitz, 2008). In short, aggregations of wild water birds were localized with the help of ornithologists; the species were identified and observed and individuals were counted 30–60 min presampling. Droppings that appeared freshly passed (within the previous hour), as judged by moisture, size, shape, and appearance of their surface, were sampled. Droppings from Anseriformes were distinguished as normal, cecal feces, and urine. From each sample, 0.5–3 ml of material was conveyed into a commercial swab system (Virocult™, MWE, Bath, UK) and mixed with the system's transport medium. Until laboratory analysis, samples were stored at 4 C to 10 C for 1–4 days.

Using the described system, cloacal and oropharyngeal swabs were concurrently collected from 1,402 hunted or captured wild birds in five administrative districts adjacent to the environmental sampling sites. Both oropharyngeal and cloacal samples were collected from each bird using a single swab (pharyngocloacal swabs). Represented species were similar to those from which environmental fecal samples were obtained (Table 1A, B). In addition, 295 dead wild birds found in these districts at that time were included (Table 1C).

A maximum of 100 mg of material from a thoroughly mixed fecal sample was transferred to 1.8 ml of Dulbecco's modified eagle (cell-culture) medium containing antibiotics (penicillin, ciprofloxacin, nystatin) and 2% (v/v) of fetal calf serum (termed sample medium). Following vortexing (1 min) and incubation at room temperature (30 min), samples were centrifuged for 2 min at 10,500 × G. Pharyngocloacal swabs were treated similarly: 1.8 ml of sample medium were pipetted into the swab tube and mixed extensively. Supernatant of up to five feces or pharyngocloacal swab samples was pooled, and RNA was extracted using the High Pure Viral Nucleic Acid Kit™ (Roche Mannheim, Germany). Examination for AIV-specific RNA by real-time reverse-transcriptase polymerase chain reaction (rRT-PCR), detecting a fragment of the matrix (M) gene of influenza A viruses, has been described (Spackman et al., 2002). An inhibition control was used with each RNA pool analyzed (Hoffmann et al., 2005). When M-positive pools were encountered, individual samples of that pool were retested and further characterized by subtype-specific rRT-PCRs (H5, H7, N1) or by generic, conventional RT-PCRs and sequencing (Phipps et al., 2004; Hoffmann et al., 2007). Virus isolation in embryonated chicken eggs was attempted from PCR-positive individual samples according to standard procedures (European Commission Decision 2006/437/EC). Descriptive statistics were carried out with the statistical software R™ (R Development Core Team, 2004).

During the study, 11 AIV RNA-positive reactions were detected from 1,991 environmental samples (Table 1A). Positives were obtained from normal feces ($n=8$), cecal feces ($n=1$), and urine ($n=2$). No viruses were isolated in embryonated chicken eggs; however, the AIV RNA amplified from two normal fecal samples of White-fronted Geese (*Anser albifrons*) from one flock could be further characterized by molecular means. The HA subtype and the pathotype were defined on the basis of a sequence of 227 nucleotides of the HA gene encoding the

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TABLE 1. Number of live-bird samples (environmental and cloacal swabs) and dead-bird specimens sampled concurrently in adjacent coastal locations in Northeastern Germany.

Species origin[a]	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Total	
								Bird Species	Bird Group[e]
A. Environmental fecal samples									
Mallard	11	47	99	137	2	63	10	369	659 (0)[b]
Eurasian Wigeon		37	37	11	27	34	1	147	
Common Teal		22						22	
Other duck species							121	121	
White-fronted Goose	20		51	36	55 (2)[c]	2		164 (2)	1,268 (11)
Bean Goose	23	31 (4)	35 (1)		46			135 (5)	
Greylag Goose		70	11	4 (1)	8	36		129 (1)	
Canada Goose				1	31	8		40	
Barnacle Goose				1		70		71	
Other goose species	24	25	53	97		39		238	
Mute Swan			37	47	77	44	50	255	
Whooper Swan			99 (1)	110 (2)				209 (3)	
Other swan species			27					27	
Black-headed Gull	10			8	1	4	1	24	64 (0)
Great Black-backed Gull		15				14	11	40	
Total	88	247 (4)	449 (2)	452 (3)	247 (2)	314	194		1,991 (11)
B. Pharyngocloacal swabs (hunter-killed birds: mallards, geese)									
Mallard		29 (1)[d]	80 (2)		2	1		112 (3)	309 (4)[b]
Other duck species	5	115 (1)	18	18			41	197 (1)	
White-fronted Goose		94	224 (1)	2		26		346 (1)	901 (3)
Bean Goose		10	169 (2)			11	42	232 (2)	
Greylag Goose			9			8	5	22	
Canada Goose						48		48	
Other goose species		5		6		1	12	24	
Mute Swan		15	5	2	3	13		38	
Whooper Swan			190	1				191	
Herring Gull		14	92 (8)				3	109 (8)	192 (8)
Great Black-backed Gull			3				38	41	
Black-headed Gull			8				34	42	
Total	5	282 (2)	798 (13)	29	5	108	175		1,402 (15)

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Table 1. Continued.

Species origin[a]	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Total	
								Bird Species	Bird Group[e]
C. Bird carcasses									
Mallard	1		5		1	3		10	29 (0)[b]
Other duck species							19	19	
White-fronted Goose		2			1			3	240 (1)
Bean Goose		1	1					2	
Greylag Goose			1					1	
Canada Goose				1	8 (1)			9 (1)	
Mute Swan	3	4	15	6	46	58	9	141	
Whooper Swan			1	1			1	3	
Other swan species			9	6	42	22	2	81	
Herring Gull	3	1	2		2	2	2	12	26 (0)
Great Black-backed Gull					1			1	
Black-headed Gull	1	2	1	3	3	3		13	
Total	8	10	35	17	104 (1)	88	33		295 (1)

[a] Mallard (*Anas platyrhynchos*), Eurasian Wigeon (*Anas penelope*), Common Teal (*Anas crecca*), Greater White-fronted Goose (*Anser albifrons*); Bean Goose (*Anser fabilis*), Greylag Goose (*Anser anser*), Canada Goose (*Branta canadensis*), Barnacle Goose (*Branta leucopsis*), Mute Swan (*Cygnus olor*), Whooper Swan (*Cygnus cygnus*), Common Black-headed Gull (*Larus ridubundus*), Great Black-backed Gull (*Larus marinus*).

[b] (Number of AIV RNA-positive samples).

[c] LPAIV H5N2 detected.

[d] Sample also positive in PCR for N1.

[e] Group comprises: ducks, geese/swans, gulls.

endoproteolytical cleavage site. A BlastN2 database search revealed two AIV of the H5 subtype as the closest relatives: A/teal/DK/64659/2003 (H5N7) and A/teal/Egypt/9865-NamRU/2005 (H5N2). The deduced amino acid sequence at the cleavage site of these viruses was -R-E-T-R-*G-L-F-, resembling a monobasic cleavage motif characteristic of Eurasian H5 subtype viruses of low pathogenicity. Further molecular characterization attempts for the other samples positive in M-specific rRT-PCR failed. However, subtypes H5, H7, and N1 could be excluded in these cases by subtype-specific rRT-PCRs. Based on the assessment of the spiked inhibition control, no PCR inhibitions were detected among the set of fecal and urine samples.

Out of a total of 1,402 pharyngocloacal swabs sampled from live, wild Anseriformes, of similar species range and spatial origin as compared to environmental fecal samples, 15 AIV RNA-positive samples were detected (Table 1B). For Anseriformes, there was no statistical difference between overall recovery results of environmental versus pharyngocloacal swab samples (11/1,927 vs. 7/1,210, $P=0.97$; Pearson's Chi-square test, not Yates-corrected). For influenza-positive samples, there was a substantial overlap of the species range, in particular White-fronted Goose and Bean Goose (*Anser fabilis*), and also in time (October to January) of detection (Fig. 1). There was a statistically significant difference between recovery results of environmental versus pharyngocloacal swab samples between ducks (0/659 vs. 4/309; $P=0.01$), but not for geese and swans (11/1,268 vs. 3/901; $P=0.17$) or gulls (0/64/ vs. 8/192, $P=0.21$; Fisher's exact 2-tailed test). It should be noted, however, that the eight positive pharyngocloacal swabs were sampled from Herring Gulls (*Larus argentatus*) at the same time and location.

Only one of 269 dead Anseriform birds collected during the study period was AIV RNA positive. No AIV RNA-positive results were detected in 26 dead gulls (Table 1C).

This study commenced 5 mo after the detection of the last of 188 cases of HPAIV H5N1 infections in wild birds in this region (FLI, 2006) and was aimed to detect possibly persisting, yet clinically silent, HPAIV in a former epicentre of infection. However, in Europe, detection of HPAIV H5N1 in wild birds has been principally restricted to carcasses, emphasizing the importance of passive monitoring for detection of this virus. Our study failed, like others (Munster et al., 2007), to detect HPAIV H5N1 in samples from live birds. This may be interpreted as evidence against a broad and permanent reservoir of this virus in wild bird populations at this juncture, but it might also indicate that such methods are inappropriate to identify small reservoir niches in these populations (Newman et al., 2007).

Active monitoring is indispensable in following the evolution of LPAIVs in their natural reservoir (Olson et al., 2006). The leading role of wild birds of the orders Anseri- and Charadriiformes as a major reservoir of LPAIV is undisputed (Munster et al., 2007). However, many species belonging to these orders are considered endangered or even at risk of extinction. Thus, at an international level, special attention is paid to the conservation of these populations and their habitats. This strongly interferes with sampling needs for AIV monitoring that focus on cloacal and oropharyngeal swabs.

Environmental samples, apart from their ease of collection, have the advantage of creating fewer disturbances to birds and their habitats, as compared to capturing or hunting. However, due to specific tissue tropisms, environmental sampling would be fundamentally disadvantageous for AIV strains that were predominantly excreted via the respiratory route. At the time of writing, compelling evidence that HPAIV H5N1 of Asian origin belongs to this group had accumulated from natural and experimental infection. Therefore, oropharyngeal–tracheal samples are considered mandatory for

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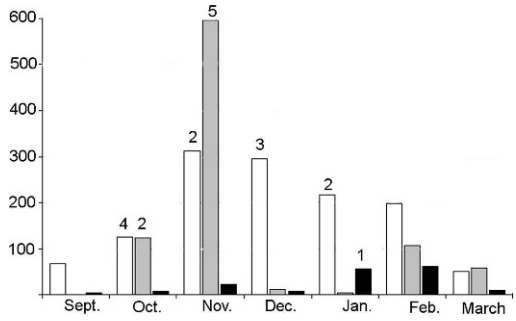


FIGURE 1. Sample numbers (y-axis) and AIV RNA-positive samples (numbers above bars) originating from Anseriforme species per month. White=environmental samples, dark gray=pharyngocloacal swab samples, black=carcasses.

screening (Cattoli and Capua, 2007). It cannot be fully excluded that the lack of detection of HPAIV H5N1 in our study stems from a failure to obtain such sample material. However, it should be kept in mind that this virus has so far been almost exclusively detected by passive monitoring; evidence for presence of the virus was not obtained from the examination of 295 dead wild birds collected during our study period in the same region.

The most overt disadvantage of such an alternative monitoring strategy seems to be an inevitable loss of species-specific information on individual samples. In a separate study (Pannwitz, 2008), we have shown that this loss can be significantly reduced if simple prerequisites are observed during sampling: Firstly, and in cooperation with ornithologists, suitable sampling sites have to be identified prior to collection. Ornithologic observation and a systematic bird count at the sampling site, 15–30 min prior to sampling, is required. The droppings from several wild bird species can be recognized from size, shape, and color, and it is possible to select only freshly dropped matter (passed approximately within <60 min prior to sampling). Thus, at least 70% of fecal and urine samples can be securely matched to a species. The bird count: sample ratio is a useful parameter for sampling intensity.

Concurrent low prevalences of LPAIV were detected in both environmental and pharyngocloacal samples of geese, swans, and gulls. The difference between duck samples cannot be easily explained and may warrant further attention. Note, however, that capturing and hunting birds for sampling may impose a bias by preferentially selecting weakened birds, thus leading to artifactually higher detection rates. Nevertheless, the overall low prevalence of AIV, and the only partially overlapping sampled populations, favor significant variations in detection rates by chance.

In conclusion, our study provides evidence that examination of environmental samples of fresh, wild-bird droppings of Anseriformes may yield similar AIV prevalence rates compared to pharyngocloacal swabs from captured and hunted birds. Still, given the low AIV prevalence in our samples, it should be kept in mind that intrinsically different types of information are collected by different sampling methods. Fecal samples, e.g., could fail to diagnose AIV in birds excreting AIV preferentially via the respiratory route. Collection of environmental avian samples is nevertheless simple, cost effective, and causes fewer disturbances to wild waterbird species and their habitats. The sample sizes and locations can be much better controlled than in hunted or captured birds. Fluctuations in AIV prevalence due to location, range of species sampled, density, and composition of wild bird populations still present a major problem with any sampling method.

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