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Authors: Camenisch, Glauco, Bandli, Risch, and Hoop, Richard

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Monitoring of Wild birds for Newcastle Disease Virus in Switzerland Using Real Time RT-PCR

Glauco Camenisch,[1,2] Risch Bandli,[1] and Richard Hoop[1] [1]Institute of Veterinary Bacteriology, National Reference Center for Poultry and Rabbit Diseases, Vetsuisse Faculty, University of Zurich, Winterthurerstrasse 270, 8057 Zurich, Switzerland; [2]Corresponding author (email: glauco.camenisch@gmail.com)

ABSTRACT: Wild birds are considered to be the natural reservoir of the Newcastle disease virus (NDV; avian paramyxovirus-1) causing Newcastle disease, and are often suspected to be involved in outbreaks in domesticated birds. To assess the epidemiologic status of wild birds living, or overwintering, in Switzerland, 3,049 cloacal swabs covering the period 2003–2006 were screened for NDV, using real time RT-PCR. All samples were negative. This result seems in contrast with previously performed serologic screenings of wild birds.

Key words: Avian paramyxovirus-1, Newcastle disease, real time RT-PCR, Switzerland, wild birds.

Although Switzerland has been officially free from Newcastle disease (ND) for more than 9 yr, continuous reports of isolated outbreaks from neighboring countries (Italy, 2000, 2003, and 2006; Austria, 2004; and France, 2005 and 2006; OIE, 2007) hint at the possibility of a future reappearance of ND. Three panzootics have been described in the last century (Alexander, 2001). Genetic characterization and subtyping of Newcastle disease virus (NDV, avian paramyxovirus-1; lineages 1 to 6 with sublineages) have revealed a complex epidemiology and a nonlinear history that involves two independent, but interacting, host systems; wild bird populations (primordial reservoir) and domestic poultry (secondary reservoir; Aldous et al., 2003; Czeglédi et al., 2006). While most of the NDV strains circulating in wild birds are lentogenic (Alexander, 2000; Globig et al., 2004), constant virus exchange between the two reservoirs (where biosecurity conditions are poor) seems to be an important factor allowing new, potentially virulent strains to emerge (Takakuwa et al., 1998; Shengqing et al., 2002; Jørgensen et al., 2004). Outbreaks of ND in wild birds are very rare; in recent years mortality related to NDV was only reported in young cormorants in North America (Allison et al., 2005).

The mobility of migratory birds and their population size (e.g., Switzerland is a wintering place for 500,000 waterfowl alone) makes them an important and not easily controllable vector of NDV dissemination (Zanetti et al., 2005). Another potentially important factor to consider is the enhancement of the wildlife–livestock interface resulting from free-range management of domestic fowl. This is particularly relevant for Swiss commercial poultry, as an important share (>40%) of the production is free-ranged. Two screening projects were initiated in Switzerland, over the last decade, to assess the prevalence of NDV in wild birds and poultry, both of which relied upon serologic tests (Schelling et al., 1999; Wunderwald and Hoop, 2002). The objective of the present study was to determine NDV infection rates in wild birds in Switzerland in order to detect possible sources of this virus to domestic poultry.

Cloacal swabs were collected, between 2003 and 2006, from four groups of wild birds. The groups included 1) Birds captured for ringing purposes in three relevant Swiss ornithologic places: Sempach Lake ($n=922$), Klingnau dam reservoir ($n=101$), and Ulmet Höchi in the Jurassic hills ($n=520$), the first two being stopover places for migrating waterfowl and the last a passage region for migratory songbirds; 2) Birds shot during waterfowl hunting seasons in several Swiss cantons ($n=306$); 3) Diseased or hurt birds from various sources (e.g., clinics, private animal shelters) ($n=384$; Baumer, 2005); and 4) Birds found dead and collected during

TABLE 1. Order, species, and numbers of tested birds; Newcastle disease virus prevalence limit estimates.

Order	Species	Sampling 2003–04	Sampling 2005–06	Total	Excluded prevalence %[a]
Anseriformes	Common Pochard *(Aythya farina)*	0	246	246	1.3
	Tufted Duck *(A. fuligula)*	11	243	254	1.2
	Mute Swan *(Cygnus olor)*	15	131	146	2.1
	Mallard *(Anas platyrhynchos)*	107	125	232	1.3
	Duck, unidentified	66	106	172	1.8
	Others	50	63	113	–
Gruiformes	Eurasian Coot *(Fulica atra)*	19	202	221	1.4
	Others	0	8	8	–
Pelicaniformes	Great Cormorant (*Phalacrocorax carbo*)	28	99	127	2.4
Charadriiformes	Black-headed Gull *(Larus ridibundus)*	3	69	72	4.1
	Yellow-legged Gull *(L. cachinnans)*	0	22	22	12.8
	Gull, unidentified	0	40	40	7.3
	Others	0	3	3	–
Podicipediformes	Great Crested Grebe *(Podiceps cristatus)*	4	45	49	6
	Little Grebe *(Tachybaptus ruficollis)*	0	4	4	–
Passeriformes	Chaffinch *(Fringilla coelebs)*	318	211	529	0.6
	Brambling *(F. montifringilla)*	4	114	118	2.6
	Great Tit *(Parus major)*	47	48	95	3.2
	Song Thrush *(Turdus philomelos)*	23	36	59	5
	Blackbird *(T. merula)*	31	42	73	4.1
	Pine Siskin *(Carduelis pinus)*	0	18	18	15.4
	Others	107	90	197	–
Accipitriformes	Buzzard spp. *(Buteo sp.)*	13	24	37	7.8
	Others	8	20	28	–
Other orders		110	76	186	–
Total		964	2085	3049	0.1

[a] 95% confidence

the extensive avian influenza (AI) monitoring campaign in winter 2005–2006 (n=816; Dalessi et al., 2006).

Most (90%) of the samples were collected during the cold season (autumn–spring), and 50% of the birds tested were juveniles (Table 1). Most (60%) of the birds were apparently healthy (e.g., captured for ringing or shot by hunters), while the remaining birds were found dead, diseased, or hurt.

Swabs were collected by ornithologists, hunters, or veterinarians and stored at 4 C and sent to the laboratory within 24 hr. Swabs were put in phosphate-buffered saline, immediately upon arrival at the laboratory, or stored at −80 C until processing. In 2003–2004, swabs were pooled, in groups of two or three, in 1.5 ml phosphate-buffered saline, of which 750 µl underwent further processing using the Ultraspec RNA isolation Kit (AMS Biotechnology, Lugano, Switzerland) following the manufacturer's instructions. In 2005–2006, swabs were processed individually with the same amount of PBS using the RNeasy Mini Kit (Qiagen AG, Basel, Switzerland), according to the instructions of the manufacturer. Extracted RNA samples were stored in RNAase-free water at −80 C.

Samples were analyzed by a TaqMan one step realtime RT-PCR assay on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, California, USA). Specific primer–probe sets for NDV M (matrix) gene (for

TABLE 2. Reference strains, pathotype, and primer–probe set specificity

Isolate[a]	Pathotype	Matrix	Fusion
APMV-1/chicken/Switzerland/Safnern/95*b*	Velogenic	+	+
APMV-1/chicken/Switzerland/Schaffhausen/96[b]	Velogenic	+	+
APMV-1/chicken/Switzerland/H404/96[c]	Lentogenic	+	−
PPMV-1/dove/Switzerland/T845/01	Lentogenic	+	−
PPMV-1/dove/Switzerland/T32/93	Lentogenic	+	−
APMV-1/chicken/NorthernIreland/Ulster/64	Lentogenic	+	−
APMV-3/turkey/England/1087/82	Not applicable	−	−
APMV-4/duck/Hongkong/D3/75	Not applicable	−	−
APMV-7/dove/Tennessee/4/75	Not applicable	−	−
APMV-8/goose/Delaware/1053/76	Not applicable	−	−

[a] APMV = avian paramyxovirus; PPMV = pigeon paramyxovirus.

[b] Field cases with high fatality in poultry.

[c] Vaccine strain.

detection of all NDV strains) and NDV F (fusion) gene (for detection of mesogenic and velogenic NDV strains) sequences were used as previously described, and validated (Wise et al., 2004). The TaqMan probes for the matrix and fusion genes were labeled 5′ Yakima-Yellow/3′ BHQ-1 and 5′ FAM/3′ TAMRA, respectively.

The following volumes were used (totaling 30 μl per well): 15 μl of 2× Master Mix without UNG (Applied Biosystems); 0.75 μl of 40x MultiScribe and RNase Inhibitor Mix (Applied Biosystems); 0.9 μl of each primer (10 μM, final concentration 300 nM); 0.75 μl of probe (10 μM, final concentration 250 nM); and 8.7 μl of RNAse-free water and 3 μl of sample, previously heated to 94 C for 5 min, and put on ice until preparation of the 96-well plate.

Thermal cycling conditions were set as follows: 30 min at 48 C for reverse transcription; 10 min at 95 C for RT deactivation and polymerase activation; and 55 cycles of 15 sec at 95 C for denaturation, followed by 1 min at 60 C for annealing and extension.

Data were analyzed with SDS Software (Applied Biosystems). Several NDV isolates (both velogenic and lentogenic strains), some of them from previously confirmed ND outbreaks in Switzerland, were used as positive controls on each assay plate. Furthermore, isolates of other avian paramyxoviruses were tested in order to check assay specificity (Table 2). Reduced test sensitivity, compared to virus isolation (probably due to the presence of PCR inhibitors in feces), has been reported (Wise et al., 2004; OIE, 2004). In order to estimate the relative sensitivity of the assay, a serial dilution of a sham-inoculated fecal sample was comparatively tested with the standard virus isolation method (OIE, 2004); the RT-PCR detected 1 median egg infective dose (EID_{50}), indicating that the two methods have comparable sensitivities.

All 3,049 samples were negative for NDV RNA. Based on these results, the maximum prevalence of NDV in the tested avian species, that cannot be excluded with the present sampling, was calculated using the program FreeCalc (Australian Veterinary Animal Health Services, Brisbane, Queensland, Australia) (Table 1; Cameron and Baldock, 1998). These negative PCR results are consistent with negative results derived from 200 routine virus isolation attempts (in eggs) on samples from wild birds that were completed in our lab between 2001 and 2005 (unpubl. data). However, results are in contrast with results from serologic testing of wild birds in Switzerland; an antibody prevalence to NDV of 10% is

reported (Schelling et al., 1999). The serologic test used in this study was not specific for NDV, so any potential cross-reactions with other avian paramyxovirus (APMV) cannot be ruled out. Considering that the viral shedding period, and thus the diagnostic window of the RT-PCR method, is much shorter than the period of persistence of antibodies, it is also possible that wild birds were infected with either low virulence, or vaccine strains, of NDV abroad (ND vaccination being prohibited in Switzerland), prior to their arrival in Switzerland.

The samples used in this study originated from a plethora of sources, and most were originally meant for avian influenza (AI) surveillance. The analogies in the epidemiology of AI and ND, as well as the considerable amount of samples, made the material suitable for this screening. However, in order to maximize its effectiveness, future monitoring should focus on collecting samples with the highest probability of virus detection. In addition to specie's factors (waterfowl and birds of prey have the highest antibody prevalence), other epidemiologic factors that must be considered, relative to NDV in wild birds, have been indicated (Stallknecht et al., 1991). These factors include age (juvenile birds show higher prevalence) and season of sampling (prevalence seems to drop from September to December).

The M gene primer–probe set was designed to detect a broad spectrum of NDV genotypes and was tested with wild bird samples (Wise et al., 2004); nonetheless, it was originally developed for virus detection in chickens, and therefore might not detect every genotype present in wildlife, or it might show varying sensitivity between bird species. This factor, in concomitance with an expected low overall prevalence of the virus, has to be carefully evaluated in interpreting the negative results. This potential limitation must be balanced with the advantages related to this method, namely speed and ease of sample collection and handling, which makes it an ideal approach for large population screening.

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