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Screening of Feral Pigeon (*Colomba livia*), Mallard (*Anas platyrhynchos*) and Graylag Goose (*Anser anser*) Populations for *Campylobacter* spp., *Salmonella* spp., Avian Influenza Virus and Avian Paramyxovirus

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Lillehaug A, Monceyron Jonassen C, Bergsjø B, Hofshagen M, Tharaldsen J, Nesse LL, Handeland K: Screening of Feral Pigeon (*Colomba livia*), Mallard (*Anas platyrhynchos*) and Graylag Goose (*Anser anser*) Populations for *Campylobacter* spp., *Salmonella* spp., Avian Influenza Virus and Avian Paramyxovirus. Acta vet. scand. 2005, 46, 193-202. –A total of 119 fresh faecal samples were collected from graylag geese migrating northwards in April. Also, cloacal swabs were taken from 100 carcasses of graylag geese shot during the hunting season in August. In addition, samples were taken from 200 feral pigeons and five mallards. The cultivation of bacteria detected *Campylobacter jejuni jejuni* in six of the pigeons, and in one of the mallards. *Salmonella diarizona* 14 : k : z53 was detected in one graylag goose, while all pigeons and mallards were negative for salmonellae. No avian paramyxovirus was found in any of the samples tested. One mallard, from an Oslo river, was influenza A virus positive, confirmed by RT-PCR and by inoculation of embryonated eggs. The isolate termed A/Duck/Norway/1/03 was found to be of H3N8 type based on sequence analyses of the hemagglutinin and neuraminidase segments, and serological tests. This is the first time an avian influenza virus has been isolated in Norway. The study demonstrates that the wild bird species examined may constitute a reservoir for important bird pathogens and zoonotic agents in Norway.

***Campylobacter; Salmonella;* avian influenza virus; avian paramyxovirus; pigeons; mallards; graylag geese.**

Introduction

The potential for spread of infectious agents from wild animals to humans and domestic animals is great, and this prospect is even more pronounced for wild birds. Many bird species play an important factor in faecal contamination of drinking water sources and agricultural crops and may also come into close contact with domestic birds enabling direct transfer of infectious agents to take place, especially when poultry are kept out of doors.

The graylag goose (*Anser anser*), a migrating bird, spends the winter in the Netherlands and Spain and usually reaches Norway by April, enroute to their summer habitats (*Andersson et al.* 2001). They habitually arrive at their first stop over, for feeding and resting, on the southwest

coast of Norway, an area that also has a high concentration of poultry breeding flocks. During the breeding season, greylag goose populations can be found in coastal areas of Mid- and Northern Norway.

In March 2003, there was an outbreak of highly pathogenic avian influenza in the Netherlands (*Parker et al.* 2003). The graylag goose is susceptible to such infections (*Perkins & Swayne* 2002) and can be a carrier of the virus (*Webster et al.* 2002). A survey in all EU countries in 2003 demonstrated low pathogenic avian influenza viruses in nine out of 3777 wild birds sampled, seven of the positives being ducks (*Brown* 2003). Some low pathogenic strains of the haemagglutinin subtypes H5 and H7 may mutate into highly pathogenic avian influenza viruses if transmitted to poultry (*Alexander* 2000).

The geese may also be infected with avian paramyxoviruses, such as Newcastle Disease virus (*Shengqing et al.* 2002). Therefore, it was of interest to know whether migrating geese were infected with any of these viruses, particularly so soon after the influenza outbreak in the Netherlands.

Some mallard (*Anas platyrhynchos*) populations can act as migrating birds with habitats in the wilderness, while others may be more or less stationary birds with more urban habitats. Ducks are particularly important in the epidemiology of avian influenza, devoid of developing clinical disease (*Hanson et al.* 2003), and they may also be infected with Newcastle Disease virus (*Stanislawek et al.* 2002, *Vickers & Hanson* 1982).

The feral pigeon (rock pigeon; *Columba livia*), has colonised the cities of Europe, and lives in close contact with humans, companion animals and domestic pigeons (*Columba livia*); which are often kept together with backyard poultry. The pigeon is particularly susceptible to avian paramyxovirus type 1 (APMV-1) (*Pennycott* 1994), including specific pigeon adapted strains (*Ujvari et al.* 2003). An outbreak of disease in autumn 2003 caused by APMV-1 was seen in fancy pigeons from the eastern part of Norway (*Løvland et al.* 2004). This was the second reported case of this disease in pigeons in Norway, the first outbreak being reported in 1984. Pigeons may also be infected with influenza virus (*Liu et al.* 2003).

The zoonotic agents *Salmonella* spp. and *Campylobacter* spp. may be carried by clinically healthy ducks, geese and pigeons (*Casanovas et al.* 1995, *Fallacara et al.* 2001, *Wahlström et al.* 2003). The aim of this study was to collect representative faecal samples from populations of migrating wild geese and stationary urban ducks and pigeons, and to examine these samples for the presence of avian influenza virus, avian paramyxovirus, *Salmonella* spp. and *Campylobacter* spp.

Materials and methods

Sampling of graylag geese

On April 3rd and 4th, 2003, 119 fresh faecal droppings were collected from eight different locations surrounding two adjacent lakes in Klepp municipality in Rogaland county. Immediately after sampling they were frozen at –70 °C, and transported frozen to the laboratory where they were kept at –70 °C, until virus cultivation or RNA isolation prior to RT-PCR.

Between August 1st and 4th, 2003, cloacal swabs were taken from 100 graylag geese carcasses on the island of Smøla. Based on body sizes and gonads , they were estimated to be 68 adults (31♂, 15♀, 22 unknown) and 32 juveniles (12♂, 11♀), 9 unknown (hatching earlier the same year). The samples were taken from within a few hours up to one day after the bird had been shot during hunting. The carcasses were swabbed in the cloacae. One of the swabs was transferred to Amies agar gel with charcoal, code 114C, from Copan Diagnostics Inc.

(California, USA) for later bacteriological examinations. A further two cotton swabs were placed in test tubes containing virus transport medium, one in an individual tube (single sample) and the other as a pool of five samples from different birds in the same tube (pooled sample). All samples were kept on ice until they reached the laboratory on the last day of sampling.

Sampling of feral pigeons and mallards

In the city of Stavanger, 100 pigeons (55 adults and 45 juveniles, hatched the same year) were caught by hand. They were swabbed in the cloacae using the same type of Amies gel swabs as used for the geese. On June 21st and 22nd, 2003, 62 birds were sampled, and the swabs were kept refrigerated at 2-8°C until June 23rd before being sent by mail to the laboratory where they were received the next day for examination. The remaining 32 samples were taken on June 29th, sent by mail to the laboratory on June 30th, and received on July 1st.

In the city of Oslo, 100 pigeons were caught by net (9) or cage traps (91) at 11 different locations, and delivered alive to the laboratory on 11 separate occasions between June 24th and September 3rd, 2003. Of these, 45 were classified by evaluation of the gonads as adults (25♂, 20♀) and 55 as juveniles (30♂, 25♀). The birds were euthanised by a blow to the head, and routine necropsy was carried out. Several samples were collected from each bird, including 3 cm of the colon for bacteriology, and two cloacal swab samples for virological investigations. Moreover, five mallards were caught by hand in Oslo, between June 25th and October 13th, 2003. Two were juveniles (2♀) and three adults (2♂, 1♀). Samples were obtained from the birds in the same way as with the graylag geese on Smøla (three cloacal swabs), and the swabs were kept on ice for up to two hours before they were frozen at –70 °C.

Permission to capture mallards and pigeons for live sampling and to euthanise pigeons was given by the Directorate for Nature Management (ref 2003/3992 ARTS-VI-ID).

Virological examinations

Faecal material from the graylag geese from Rogaland, and the pooled swab samples from the other bird populations (except for the pigeons from Stavanger), were inoculated in embryonated hen eggs. Mortality of embryos was registered daily for one week, after which the embryos in the eggs, if no mortality was observed, were killed by chilling (*Versteeg* 1985). The allantoic fluid was collected, and a hemagglutination (HA) test was performed. The allantois from the eggs where embryonic death was observed within a week was used for a second passage in embryonated eggs, in addition to the performance of an HA test.

RNA isolation was performed on the same geese faecal samples and the individual cotton swabs (single samples), prior to testing by RT-PCR. All the samples from the mallards were frozen and the inoculation of embryonated eggs was carried out after the RT-PCR had been performed on two of the samples. RNA isolation was carried out on a 140 µl sample, using the QIAamp Viral RNA mini kit according to the manufacturer's instructions (Qiagen, the Netherlands). A RT-PCR, for the detection of avian paramyxovirus 1, was performed on the samples using primer sequences (MSF1: 5'-GAC CGC TGA CCA CGA GGT TA-3' and MSF2: 5'- AGT CGG AGG ATG TTG GCA GC- 3') obtained from the Weybridge Reference Laboratory, spanning the cleavage site of the fusion protein (Ian Brown, Personal Communication). A RT-PCR, for the detection of influenza A virus, was performed using general influenza A primers designed in the M-gene segment (*Fouchier et al.* 2000).

Both RT-PCR tests were performed in a two-

step protocol, using Superscript III (Invitrogen) for reverse transcription, and Hotstar Taq DNA polymerase (Qiagen) for PCR. The reverse transcription was performed at 50ºC for 45 min for the avian paramyxovirus detection, and at 55ºC for 30 min for the avian influenza detection. Both reactions were followed by inactivation of the reverse transcriptase at 70ºC for 15 min prior to PCR. The amplification programs consisted of an initial polymerase activation step for 15 min at 95 °C, followed by 40 cycles with the following conditions for the avian paramyxovirus PCR protocol: 94 °C for 40 s, 55 °C for 20 s and 72 °C for 60 s, and by 45 cycles with the following conditions for the avian influenza protocol: 94 °C for 45 s, 58 °C for 45 s and 72 °C for 30 s. A final elongation step at 72 °C for 5-10 min was performed, followed by chilling to 4ºC. The concentration of Mg2+ in the reactions was 1.5 mM for both protocols, and Qiagen's Q solution was added in the PCR for avian paramyxovirus detection. Amplified products were separated by gel electrophoresis and visualised by UV illumination of the gel stained with ethidium bromide.

Cultivation of bacterial pathogens

Examination for the presence of *Campylobacter* spp. was carried out on all the fresh samples (those that had not been frozen) from the pigeons from Stavanger and Oslo, the mallards, and the geese from Smøla, but not the geese faecal samples from Rogaland. The fresh samples were cultivated directly on CAT-agar: *Campylobacter* blood free selective agar (Oxoid CM 739, Oxoid, UK) (*Aspinall et al.* 1996) supplemented with cefoperazone, amphotericin B and teicoplanin (Oxoid SR 174), and incubated in a microaerophilic atmosphere at 37 °C for 2-3 days. Presumptive *Campylobacter* spp. colonies were tested for typical appearance and motility by phase contrast microscopy and subcultured on 5% bovine blood agar under the same conditions as described above. The different species were identified by phenotypic assays, including growth pattern at 42ºC, catalase production and hippurate hydrolysis.

The same samples were tested for the presence of *Salmonella* spp. by use of the Nordic Committee on Food Analyses (NMKL) method no. 71, which has been validated and is considered equivalent to ISO no. 6579:1993, and revised editions, according to the EU Commission Decision 97/278/EC. The principle for the method is non-selective pre-enrichment in phosphate buffered peptone water, selective enrichment in Rappaport-Vassiliadis soya peptone broth, and plating out on red violet bile agar plates. Colonies of presumptive *Salmonella* spp. were subcultured on lactose sucrose bromthymol blue agar plates and tested for hydrogen sulfide production on triple sugar/iron (TSI) agar and urease production on urea agar. Hydrogen sulfide positive and urease negative isolates were further tested by API20E (bioMérieux, Marcy l'Etoile, France).

Sequence analysis

The PCR products were sequenced using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1 (Applied Biosystems, California, USA) according to manufacturer's instructions, and analysed on an ABI PRISM 3100-*Avant* Genetic Analyzer (Applied Biosystems).

The software used for sequence analysis was Sequencher™ version 4.1.4 (Gene Codes Corporation, http://www.genecodes.com), Clustal W Multiple Sequence Alignment Program version 1.83 (http://www.ebi.ac.uk/clustalw/), BioEdit Sequence Alignment Editor version 5.0.9 (Tom Hall, Department of Microbiology, North Carolina State University, North Carolina, USA, http://www.mbio.ncsu.edu/BioEdit/bioedit.html), the Influenza Sequence Database at LANL, http://www.flu.lanl.gov/

(*Macken et al.* 2001), PHYLIP Package version 3.6 (Joe Felsenstein, Department of Genome Sciences, University of Washington, Seattle, Washington, USA, http://evolution.gs.washington.edu/phylip.html), and TreeView (Win32) version 1.6.6 (Roderick D. M. Page, Division of Environmental and Evolutionary Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, UK, http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Results

An apparently healthy adult male mallard, sampled in an Oslo river during October 2003, was found to be influenza A virus positive; evidenced by RT-PCR. The virus was passaged twice in embryonated eggs, and mortalities of embryos were observed in four of five eggs during the second passage. An HA test was performed on allantoic fluid, evidencing HA activity. The virus was then run in a hemagglutination-inhibition test with sera against H5N1, H5N2, H5N3, H7N1, H7N3 and H7N7, as well as against avian paramyxovirus strains (APMV-1, APMV-2 and APMV-3). No inhibition of hemagglutination was observed with any of the sera. RT-PCR and sequencing of the hemmaglutinin segment was performed using primers designed to amplify all hemagglutinin subtypes of influenza A virus (*Hoffman et al.* 2001). The virus was found to be an H3 subtype, and the complete H3 gene of the duck isolate was further amplified and sequenced using subtype specific primers. Additionally, the neuraminidase gene was amplified, using primers designed to amplify all known neuraminidase subtypes of influenza A virus (*Hoffman et al.* 2001), sequenced, and found to be of the N8 subtype. Genbank accession numbers AJ841293 and AJ841294 have been assigned for the hemagglutinin and neuraminidase genes respectively and the results of the phylogenetic analyses performed on the whole coding part of both genes are shown in Fig.1a and Fig. 1b. The virus isolate was designated A/Duck/Norway/1/03.

An allantoic fluid sample containing the virus was sent to the EU reference laboratory for avian influenza in Weybridge, UK, and was confirmed by serological tests to be an H3N8 isolate.

Neither avian influenza virus nor avian paramyxovirus were detected in any of the 119 faecal samples from graylag geese (fresh droppings and swabs). Furthermore, all 100 samples from pigeons tested by inoculation in embryonated eggs and by RT-PCR were negative for these avian viruses.

The cultivation of bacteria from the cloacal swabs of 100 pigeons from Stavanger, revealed *Campylobacter jejuni jejuni* in four samples (two young and two adults). *C. jejuni jejuni* was identified in two of the 100 pigeon samples from Oslo, both being males, one juvenile and one adult, as well as in one of the five mallards, an adult female. No pigeons or mallards were positive for *Salmonella. Campylobacter* spp. was not found in any of the 100 geese samples from Smøla. However, *Salmonella diarizona* 14 : k : z53 was detected in one adult goose.

Discussion

During this screening study, avian influenza virus was isolated for the first time in Norway. In total, 324 samples were investigated for the presence of avian paramyxovirus and avian influenza virus, and influenza A virus H3N8 was identified in one mallard (A/Duck/Norway/1/03). All the other samples tested negative for both agents. A/Duck/Norway/1/03 was found to be closely related to the Eurasian avian lineage of H3N8 type (*Saito et al*. 1993, *Bean et al.* 1992), as shown in Figs. 1a and 1b, branching off near the root of the lineage, despite recent sampling, indicating a long-established equilibrium between the virus and its natural

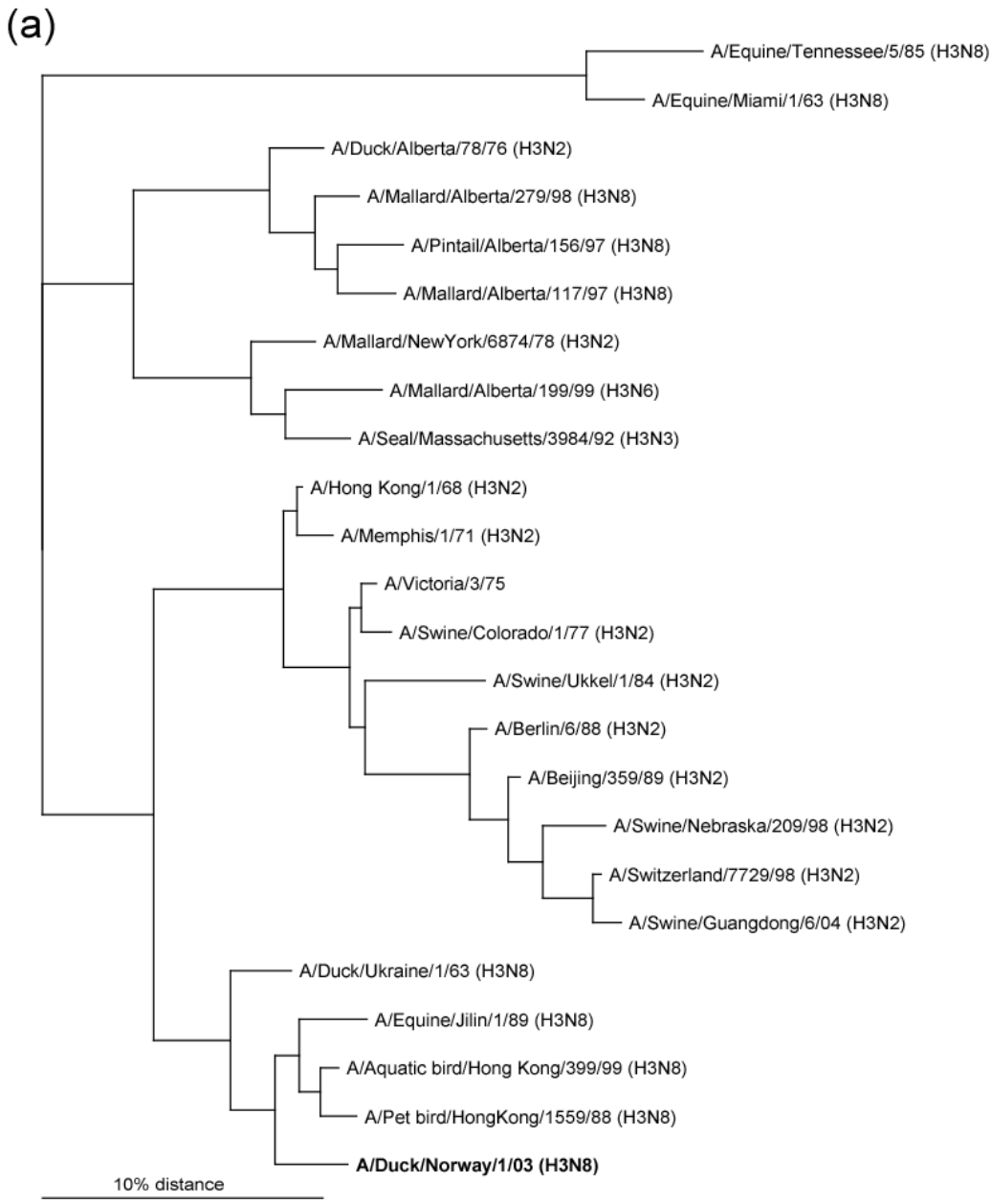
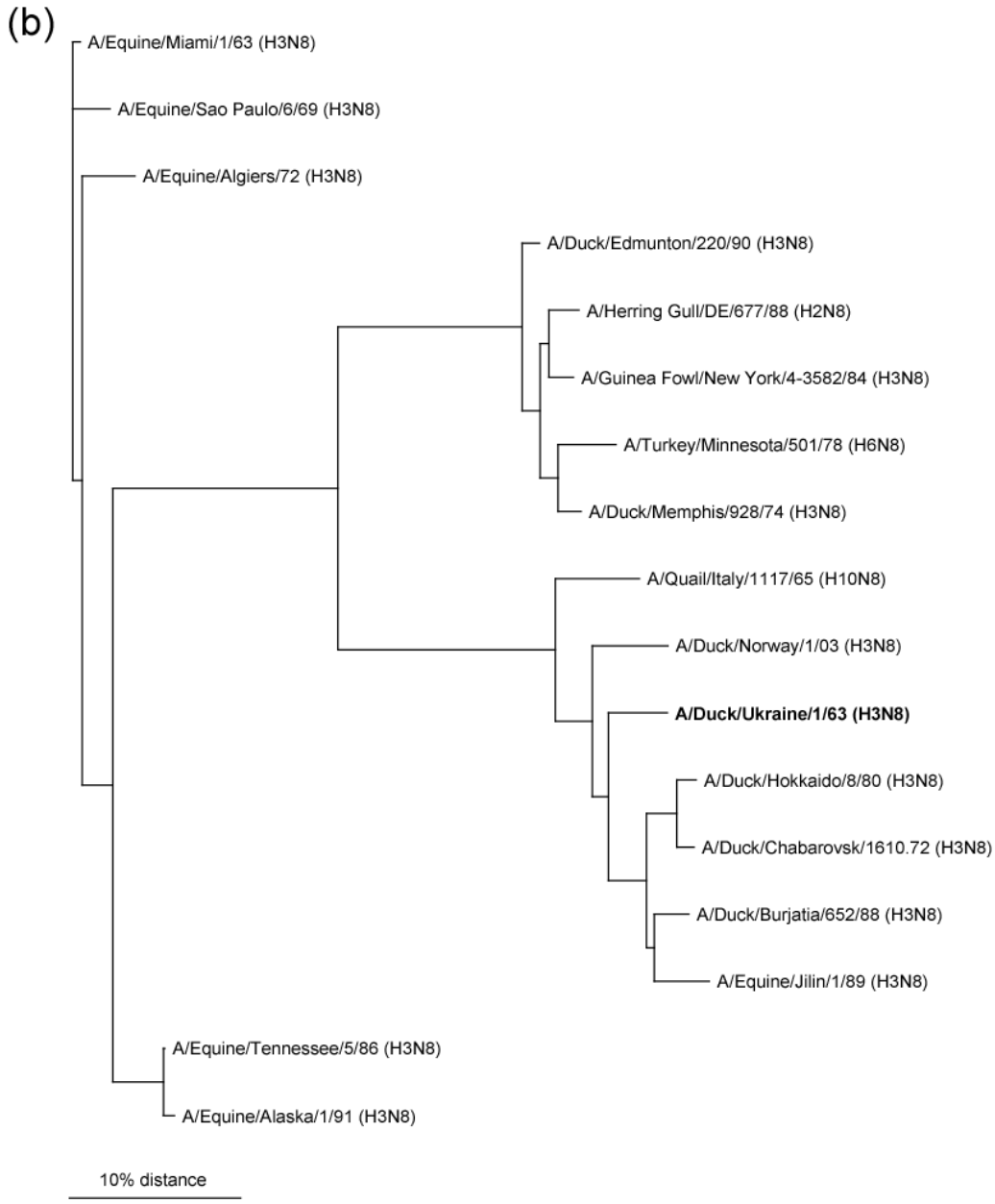


Figure 1. Phylogenetic analysis of the influenza A/Duck/Norway/1/03 isolate based on the hemagglutinin (a) and the neuraminidase (b) genes, using maximum likelihood tree construction method.


(b)
A/Equine/Miami/1/63 (H3N8)
A/Equine/Sao Paulo/6/69 (H3N8)
A/Equine/Algiers/72 (H3N8)
A/Duck/Edmunton/220/90 (H3N8)
A/Herring Gull/DE/677/88 (H2N8)
A/Guinea Fowl/New York/4-3582/84 (H3N8)
A/Turkey/Minnesota/501/78 (H6N8)
A/Duck/Memphis/928/74 (H3N8)
A/Quail/Italy/1117/65 (H10N8)
A/Duck/Norway/1/03 (H3N8)
A/Duck/Ukraine/1/63 (H3N8)
A/Duck/Hokkaido/8/80 (H3N8)
A/Duck/Chabarovsk/1610.72 (H3N8)
A/Duck/Burjatia/652/88 (H3N8)
A/Equine/Jilin/1/89 (H3N8)
A/Equine/Tennessee/5/86 (H3N8)
A/Equine/Alaska/1/91 (H3N8)
10% distance

host, as proposed earlier (*Bean et al.* 1992, *Horimoto & Kawaoka* 2001).

Influenza A viruses are widely distributed in duck populations throughout the world (*Alexander* 2000). In Northern Europe, *Fouchier et al.* (2003) found 1.4% positives in geese and 2.6% in ducks by RT-PCR sampled in the Netherlands, in Sweden, on the Faeroe Islands and in Iceland. According to reports from EU member states, low pathogenic strains have been detected in wild birds and domestic ducks as far north as Denmark (*Alexander & Manvell* 2003). Our finding demonstrates that there is a theoretical possibility, even in Norway, of transmission of low pathogenic avian influenza virus between waterfowl and poultry. If such viruses are of the H5 or H7 subtypes, fowl plague could develop in poultry populations (*Alexander* 2000). Furthermore, poultry may also transmit the virus to humans (*Webby & Webster* 2003).

This screening of faecal samples from graylag geese, mallards and pigeons indicates that these birds do not constitute an important source of infection for zoonotic agents like *Salmonella* and *Campylobacter. Salmonella diarizona* was found in one graylag goose, the only *Salmonella sp.* found in the 305 bird samples tested. This particular *Salmonella* subspecies very rarely causes disease in humans (*Hall & Rowe* 1992), but it has been isolated relatively frequently from sheep faeces, and it has been associated with abortions in sheep in Northern Norway (*Mork et al.* 1994). However, the serotype *S. diarizona* 14 : k : z53, that was found in the goose, has never been isolated from sheep.

Campylobacter jejuni jejuni was found in seven samples; four pigeons from Stavanger, two from Oslo (3.0 % of all pigeons tested) and one out of five mallards from Oslo. These findings are of the same magnitude as in a previous survey (*Kapperud & Rosef* 1983) where 4.2 % of the tested pigeons in Oslo were positive for *Campylobacter* spp. Several avian species were included in this previous study, and *Campylobacter* was also found in birds such as crows and gulls. Moreover, *Salmonella* was found in gulls. However, species of the order *Anseriformes* (comprising ducks and geese) were not included in that study. *Wahlström et al.* (2003) found thermophilic *Campylobacter* in 15% of Canada geese sampled, but no salmonellaes in a Swedish study.

The results of the present study demonstrate that the wild bird species included may constitute a reservoir for important pathogens that could be of risk to other birds, farm animals and humans in Norway. The relatively low prevalences found, however, indicate that they are not important sources of such infectious agents. The numbers of individuals tested were low, particularly for mallards, in which both influenza virus and *Campylobacter* was found. The intention was to catch 100 birds in all populations sampled, but the capturing of mallards was not very successful. All the samples from geese and half of the pigeon samples had to be transported to the laboratory for examination, and factors like sunlight (UV radiation), temperature variations and the time period elapsing between sampling and examination may have influenced the outcome. The similarities in results between the Oslo and Stavanger pigeon populations do, however, indicate that these parameters did not hamper the bacteriological results significantly. Still, comprehensive studies of these and other bird species are necessary in order to collect sufficient knowledge regarding avian wildlife reservoirs for zoonotic agents and animal pathogens.

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Sammendrag

Screening av byduer (Colomba livia), *stokkender* (Anas platyrhynchos) *og grågås (Anser anser) for* Campylobacter *spp.,* Salmonella *spp., aviært influenza virus og aviært paramyxovirus.*

Til sammen 119 ferske avføringsprøver ble samlet fra grågås på trekk nordover i april, og kloakksvabere ble tatt fra 100 skrotter av samme fugleart som ble skutt under jakta i august. I tillegg ble det tatt prøver av 200 byduer og fem stokkender. Ved bakteriedyrking ble *Campylobacter jejuni jejuni* funnet hos seks av duene og hos en av stokkendene. *Salmonella diarizona* 14 : k : z53 ble funnet hos ei grågås, mens alle duene og stokkendene var negative for salmonella. Aviært paramyxovirus ble ikke påvist i noen av de undersøkte prøvene. Ei stokkand fra ei elv i Oslo ble funnet positiv for influensa A virus med RT-PCR og ved innokkulering i embryonerte egg. Isolatet som benevnes A/Duck/Norway/1/03, ble funnet å være av H3N8 type, basert på sekvensanalyser av hemagglutinin og neuraminidase genene, og ved serologiske undersøkelser. Dette er første gang aviært influensavirus har blitt isolert i Norge. Studien viser at de ville fugleartene som ble undersøkt kan utgjøre et reservoar for viktige fuglepatogene og zoonotiske agens i Norge.

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