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ORIGINAL PAPER

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Do Canada geese (*Branta canadensis* Linnaeus, 1758) carry infectious agents for birds and man?

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Abstract Currently, large groups of Canada geese (*Branta canadensis* Linnaeus, 1758) aggregate in recreational areas of north-western Germany. Questions have arisen as to whether these birds represent a special risk factor as a source of zoonotic agents for humans and as a source of viruses, causing notifiable or reportable diseases, for domestic poultry and waterfowl. To answer these questions, a total of 289 eggs were collected in 2002 and 2003 on a recreation site and assayed. *Chlamydia psittaci* was not isolated and neither was chlamydial antigen detected by polymerase chain reaction. All virus-isolation attempts were unsuccessful. Neither *Salmonella* spp. nor *Campylobacter* spp. was isolated from embryonic tissues, chorioallantoic membranes or yolk-sac membranes. The presence of antibodies against Newcastle disease virus and influenza A virus (haemagglutinin subtypes H5 and H7) was demonstrated in egg yolk. Antibodies were also detected against the egg-drop syndrome 1976 and duck plague viruses. It is concluded that further surveillance studies are needed for a reliable risk assessment.

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Introduction

The Canada goose (*Branta canadensis* Linnaeus, 1758) lives in wetlands in the northern parts of America and Europe in large numbers (Grömping 2003). Owing to the lack of natural predators and due to the obvious suitability of fresh-water lakes surrounded by meadows for the geese, large populations of Canada geese have developed over the years in the northern part of Germany. Since most of the lakes are visited frequently by people for recreational purposes, questions arise as to the possible risk of transmission of zoonotic agents from these birds to man. It is currently unknown whether influenza A and paramyxoviruses are carried and shed by free-living Canada geese; eggs were collected in the study area and examined.

Influenza A viruses of various subtypes were described in waterfowl (Hergarten 1994; Swayne and Halvorson 2003). At least circumstantial evidence suggests that ducks and geese were the source of infection for chickens and turkeys during the catastrophic outbreaks of highly pathogenic avian influenza in Italy (1999–2002), in Belgium, The Netherlands and Germany (2003), and in many south-east Asian countries (2004). The evidence for links between wild bird populations and epidemic disease outbreaks in domestic poultry provides substantial reasons for a more sophisticated, large-scale and persistent surveillance of wild birds for influenza A and avian paramyxoviruses.

Avian paramyxoviruses (aPMV) of any virulence were also detected in waterfowl, causing predominantly subclinical infections in most of these different bird species (Kaleta and Baldauf 1988; Alexander 2003). Spread from persistently infected waterfowl to domestic chickens and

turkeys, aPMV have appeared in the past with disastrous consequences for egg-laying chickens and meat-producing chickens and turkeys (Lancaster and Alexander 1975; Alexander 2003). The serotypes 4 and 6 of avian paramyxoviruses are, besides serotype 1, the most frequently isolated viruses of the genus (Alexander 2003).

Egg-drop syndrome 1976 virus is present in many species of waterfowl without obvious signs of disease (McFerran and Adair 2003; Kaleta et al. 2003). Lateral spread to chickens, and possibly also to other avian species, results in thin-shelled or shell-less eggs and severe drops in egg numbers (Kaleta et al. 2003).

Chlamydia psittaci is a major zoonotic agent that is horizontally transmitted from a large variety of bird species to man. Shore birds, gulls and other waterfowl have been found to be frequently infected and play a major role as a source of infection in human cases of chlamydiosis (also known as psittacosis or ornithosis). Chlamydophila (*Chlamydia psittaci*) was detected in many species of waterfowl including the Canada goose and eggs from this species (Wilt et al. 1972; Kaleta and Taday 2003).

The aim of this study is to report on results of examinations of embryonic tissues from Canada geese for the presence of bacteria, fungi and viruses that might be of concern for a number of reasons: (1) as causes of waterfowl diseases, (2) as causes of diseases that might spread to domestic poultry and waterfowl, and (3) as agents that might be transmitted to humans and initiate zoonotic disease. Egg yolk of the same eggs was used to check for antibodies against major disease-causing agents.

Fig. 1 Map of Germany showing the federal states and the study area (Bergisch-Gladbach) in the state of North Rhine-Westphalia (*shaded area*). (Courtesy of R. Boy)

Materials and methods

With the permission of the responsible authorities in charge of the protection of natural resources of flora and fauna, a total of 107 eggs were collected in spring 2002 from free-living healthy appearing Canada geese living in the vicinity of a fresh-water lake in the north-western part of Germany; the lake is used regularly for recreational purposes (Fig. 1). In the spring of 2003, a total of 182 eggs were collected at the same site. The global population of Canada geese has been estimated to be in the range of 3 million birds (Carboneras 1991); the exact figure for the European non-migrating population in the particular area from which the eggs came is estimated to be in the range of 80–100.

External examination of eggs

All eggs were marked, weighed and measured (length and width), and external shell properties such as shell colour, surface structure and traumatic defects were recorded (Scholtyssek 1978). Fertilized eggs were checked for living or dead embryos by candling using a UV-lamp. Eggs containing living embryos were further incubated at 37.5 °C in a moist atmosphere and turned twice daily until processing (Siegmann 1993).

Internal examination of eggs

Eggs were opened at the air space, and embryos were removed and examined for size and physical abnormalities (Brown 1988; Siegmann 1993). Their weight and body length were determined. During dissection, internal organs (liver, spleen, kidney, intestines) were removed for bacteriological, mycological, and virological assays (Bönner et al. 2003).

Bacteriological examination

Undiluted yolk was used for culturing bacteria, particularly *Salmonella* spp., by placing approximately 1 ml of yolk in 50 ml of enrichment broth, with subsequent incubation for 48 h at 41 °C and subculturing on brilliant-green phenol-red lactose saccharose agar (BPLS) and multified semisolid Rappaport-Vassiliadis (MSRV) agar plates (Quinn et al. 1994; ISO 6579). *Escherichia coli*, *Pasteurella multocida* and *Campylobacter* spp. were also cultured. Ovine blood agar and BPLS agar plates were used to culture bacteria from internal embryonic organs (trachea, lung, liver, spleen, kidney, and yolk-sac membrane).

Mycological examination

Kimmig agar plates were used for the isolation of fungi from yolk and internal embryonic organs. Typing and species diagnosis of the isolates followed standard procedures (Kunkle and Richard 1998).

Tests for Chlamydia spp.

For the isolation of *Chlamydia* spp., HeLa cell cultures were inoculated with organ homogenates, incubated for 3 days, subcultured twice in these cells and stained with methylene-green-eosin stain (Gimenez 1964). In an attempt to detect chlamydial antigen(s), a polymerase chain reaction (PCR) was applied to organ homogenates and lysed HeLa cells of the first passage using the methods described by Kaltenböck et al. (1991) and Yoshida et al. (1998).

Virological examination

Yolk samples and embryonic tissues were tested for the presence of avihepadnavirus according to a PCR protocol provided by Pult et al. (2001) that detects all known avihepadnaviruses. For positive controls, yolk was mixed with a duck hepatitis B virus (HBV) suspension that contained 10^3 HBV-DNA molecules.

Homogenized tissues derived from trachea, lung, liver, spleen, kidney plus pronephros, yolk-sac membranes and chorioallantoic membranes were inoculated onto primary chicken embryo liver cell cultures (CELC) for virus-isolation purposes (Schat and Purchase 1998). Cultures showing cytopathic changes were inoculated into the allantoic cavity of approximately 10-day-old embryonated chicken eggs.

Serological examination of egg yolk

Yolk samples [1 ml yolk in 3 ml phosphate-buffered saline (PBS)] were collected from each egg. Diluted yolk was checked for antibodies against avian influenza A viruses of the haemagglutinin subtypes H5 and H7 (Swayne et al. 1998), paramyxoviruses types 1, 4 and 6 (Alexander 1998), egg-drop syndrome 1976 virus, an avian adenovirus (McFerran 1998) in haemagglutination-inhibition tests according to standard procedures (EC directive 92/40; OIE Manual 2000). Antibodies against duck plague virus (*Anatid herpesvirus* 1) were assayed in virus-neutralization tests (Woolcock 1998).

Results

Egg-shell quality and secondary-taint eggs

Egg-shell abnormalities or cracked shells are a rare event. Only 7 out of 107 eggs in the year 2002 and 7 out of 182 eggs in the year 2003 were affected with shell abnormalities. Further results on morphometric data are described elsewhere (Bönner et al., in preparation).

Embryonation of eggs

All 289 eggs were collected from natural nesting places of healthy appearing Canada geese and were examined for various stages of embryo development. Out of 107 eggs obtained during the 2002 collection period, 73 eggs were in various stages of embryonic development, 15 eggs were either not fertilized or not yet incubated to a stage where embryos could be seen, and 19 eggs contained dead-in-shell embryos. In 2003, a total of 182 eggs were collected. Out of these, 50 eggs were either not fertile or not yet incubated, 87 eggs were embryonated, and 45 eggs contained dead-in-shell embryos. The time interval between death of these embryos and the time of investigation could not be readily determined.

The embryonic mass varied up to a maximum of 93 g. Four embryos that were derived from different clutches displayed malformations of the beak, cervical vertebrae and femur bone.

Isolation of bacteria

Attempts were made to isolate bacteria from a total of 289 samples of egg yolk; 160 alive and 64 dead-in-shell embryos and 160 embryonic membranes were analysed for bacteria that might affect embryonic development or might be of importance as pathogens for other bird species or for humans. Table 1 contains the results of bacteriological examination of internal organs, chorioallantoic membranes and yolk sacs of fertile eggs with living embryos, infertile or not incubated eggs and eggs containing dead-in-shell embryos. *Staphylococcus* spp. and *Bacillus* spp. were isolated from eggs of all three groups. None of the eggs contained *Salmonella* spp. or *Campylobacter* spp. *Pasteurella* spp. were isolated from one egg, and *Lactobacillus* spp. were isolated from four eggs. *Escherichia coli*, *Pseudomonas aeruginosa* and *Vibrio fluvialis* were cultured once from a dead-in-shell embryo.

Isolation of *Chlamydia* spp.

For the detection of *Chlamydia* spp., the same tissues plus egg-yolk membranes were employed as for other bacteriological examinations. *Chlamydia* spp. was neither isolated in HeLa cell cultures nor was chlamydial antigen detected by polymerase chain reaction (PCR) in embryonic tissues, chorioallantoic membranes or egg-yolk membranes in eggs obtained during the 2002 or 2003 collection periods.

Table 1 Isolation of bacteria from organs, chorioallantoic membranes (*CAM*) and yolk-sac membranes (*YSM*) of eggs or embryos collected in 2002 and 2003

Group and number of eggs or embryos per year	Isolated bacteria	Number of isolates[a] from		
		Organs 2002/2003	CAM 2002/2003	YSM 2002/2003
Eggs not fertilized, or not yet incubated, in year				
2002 = 15 eggs	*Staphylococcus* spp.	0/0	0/0	0/1
2003 = 50 eggs	*Bacillus* spp.	0/0	0/0	0/2
Fertile eggs with living embryos, in year				
2002 = 73 eggs	*Staphylococcus* spp.	23/5	22/0	19/2
2003 = 87 eggs	*Streptococcus* spp.	0/1	0/0	0/0
	Bacillus spp.	5/3	6/0	6/0
	Lactobacillus spp.	0/3	1/1	0/1
	Escherichia coli	1/0	2/0	2/0
	Pasteurella spp.	1/0	1/0	1/0
Dead-in-shell embryos, in year				
2002 = 19 eggs	*Staphylococcus* spp.	0/3	1/6	1/4
2003 = 45 eggs	*Lactobacillus* spp.	0/1	0/0	0/0
	Bacillus spp.	0/4	1/2	1/1
	Pseudomonas aeruginosa	0/1	0/0	0/0
	Vibrio fluvialis	0/1	0/0	0/0
	Escherichia coli	0/1	0/0	0/0

[a]Some samples yielded more than one isolate

Isolation of fungi and yeasts

A total of 50 non-fertile, 160 fertile and 64 dead-in-shell eggs were cultured for moulds and yeasts. Moulds and yeasts were only infrequently isolated from eggs and internal tissues as shown in Table 2. *Aspergillus fumigatus, Penicillium* spp. and *Mucor* spp. were cultured only from eggs containing embryos that were alive at the time of investigation. None of the eggs yielded yeasts.

Isolation of viruses

Embryonic tissues derived from 160 eggs were examined for cytopathogenic viruses. Neither in eggs collected during 2002 nor in eggs collected during 2003 were any viruses isolated in chicken embryo liver cell cultures (CELC) from internal embryonic organs and chorioallantoic membranes. In addition, attempts to demonstrate the presence of avian hepadnavirus DNA by polymerase chain reaction failed in all 160 attempts.

Serological examination of egg yolk

Antibodies against Newcastle disease virus (paramyxovirus serotype 1) were detected in the haemagglutination-inhibition (HI) test in yolks of eggs sampled in both years (Table 3). In egg-yolk samples from 2002, 7 out of 107 eggs (6.5%) contained antibodies against Newcastle disease virus. In the egg-yolk samples of 2003, 15 out of 182 eggs (8.2%) had antibodies against Newcastle disease virus. The HI titres were higher in samples collected in the year 2002 (mean $\log_2 = 5.4$) than in 2003 (mean $\log_2 = 3.8$).

HI antibodies against paramyxovirus (PMV) serotype 4 were not detected in any of the 289 egg-yolk samples. Whereas 4 out of 107 egg-yolk samples collected during 2002 contained antibodies against PMV-6, none of the 182 2003 egg-yolk samples contained antibodies against PMV-6.

HI antibodies against influenza A virus of the haemagglutinin subtype H5 were detected in one out of 107 eggs in the 2002 sampling period but not in any of the 181 2003 samples.

Table 2 Isolation of moulds and yeasts from embryonic organs, chorioallantoic membranes (*CAM*) and yolk-sac membranes (*YSM*) of eggs collected in 2002 and 2003

Group of eggs or embryos	Isolated fungi and yeasts	Number of isolates[a] from		
		Organs 2002/2003	CAM 2002/2003	YSM 2002/2003
Eggs not fertilized, or not yet incubated, in year				
2002 = 15 eggs	None, sterile	15/50	15/50	15/50
2003 = 50 eggs				
Fertile eggs with living embryos in year	None, sterile	68/87	72/87	70/87
2002 = 73 eggs	*Aspergillus fumigatus*	0/0	0/2	0/0
2003 = 87 eggs	*Penicillium* spp.	3/0	2/0	3/0
	Mucor spp.	0/0	1/0	1/0
	Unidentified fungi	3/0	0/1	1/0
	Unidentified yeasts	0/0	0/0	0/0
Dead-in-shell embryos	None, sterile	19/45	19/45	19/45
2002 = 19 eggs				
2003 = 45 eggs				

[a]Some samples yielded more than one isolate

Table 3 Assays for antibodies in egg yolk

Virus	Year	Number of positives/total	Number of yolk samples with HI titre (log_2)							
			≥1	2	3	4	5	6	7	≥8
Newcastle disease virus	2002	9/107	0	0	0	2	3	3	0	1
	2003	15/181	0	4	7	5	2	1	0	0
Paramyxovirus type 4	2002	0/107	0	0	00	0	0	0	0	0
	2003	0/181	0	0	0	0	0	0	0	0
Paramyxovirus type 6	2002	4/107	0	0	1	3	0	0	0	0
	2003	0/181	0	0	0	0	0	0	0	0
Influenza A virus, H5	2002	1/107	0	0	0	1	0	0	0	0
	2003	0/181	0	0	0	1	0	0	0	0
Influenza A virus, H7	2002	2/107	0	0	0	1	1	0	0	0
	2003	0/181	0	0	0	0	0	0	0	0
Egg-drop syndrome 1976 virus	2002	20/107	0	0	3	8	7	1	1	0
	2003	37/181	0	8	12	14	3	0	0	0
Duck plague virus	2002	14/107	0	0	0	4	3	4	2	1
	2003	0/181	0	0	0	0	0	0	0	0

HI antibodies against influenza A virus of the haemagglutinin subtype H7 were detected in two yolk samples from 2002. All 181 egg-yolk samples from 2003 were free from detectable antibodies against influenza A virus of the subtype H7.

HI antibodies against the egg-drop syndrome 1976 virus were detected in 20 out of 107 (18.7%) yolk samples collected in 2002 and in 37 out of 181 (20.4%) samples collected in 2003.

Table 4 shows the relationship between embryonic development and antibody status against Newcastle disease virus and egg-drop syndrome 1976 virus.

Virus-neutralizing antibodies against duck plague herpesvirus (i.e. duck viral enteritis virus) were assayed in chicken embryo cell cultures. A total of 14 out of 107 (13.1%) egg-yolk samples from eggs collected in 2002 contained neutralizing antibodies. No neutralizing antibodies against duck plague herpesvirus were detected in yolk samples derived from 2003.

Discussion

The most significant finding in this study is the demonstration of antibodies against Newcastle disease virus (NDV), the causative agent of epidemics that are notifiable in most countries. The detection of antibodies in egg yolk proves that the Canada goose is susceptible to this virus and may serve as carrier and as shedder of NDV (Kaleta and Baldauf 1988). It appears from experimental studies with domestic geese that birds of the order Anseriformes are somewhat resistant to infection with NDV (Bolte et al. 2001). The presence of antibodies in egg yolk is therefore an indication of a heavy infection. Waterfowl is thought to be the main and most effective source of Newcastle disease in domestic chickens and turkeys (Telbis et al. 1989; Bolte et al. 2001; Alexander, 2003). Horizontal spread of NDV from infected waterfowl to domestic birds is incriminated in many epidemics in recent years (Alexander 2003). The Canada goose is a neozoon bird species that ceased migration in European countries. Consequently, long-distance spread of viruses is unlikely.

It is also important to note that antibodies against influenza A virus of the haemagglutinin subtypes H5 and H7 were detected in yolk of eggs sampled in 2002. This fact proves that Canada geese are susceptible to influenza A viruses and may be a risk factor if living close to domestic poultry (Easterday et al. 1968; Rosenberger et al. 1974; Nettles et al. 1985; Stallknecht and

Table 4 Relationship between embryonic development and antibody status against Newcastle disease virus (NDV) and egg-drop syndrome 1976 (EDS) virus

Test virus	Embryo status	Year	Number of egg yolk samples with HI titre (log_2)						
			2	3	4	5	6	7	8
NDV	Not fertile or not yet incubated eggs	2002	0	0	2	3	3	0	1
		2003	0	2	0	0	0	0	0
	Live embryos of variable size	2002	0	0	0	0	0	0	0
		2003	4	5	3	0	0	0	0
	Dead-in-shell embryos	2002	0	0	0	0	0	0	0
		2003	0	0	2	2	1	0	0
EDS	Not fertile or not yet incubated eggs	2002	0	0	0	0	0	0	0
		2003	2	3	6	0	0	0	0
	Live embryos of variable size	2002	0	3	8	7	1	1	0
		2003	5	8	6	2	0	0	0
	Dead-in-shell embryos	2002	0	0	0	0	0	0	0
		2003	0	2	2	1	0	0	0

Shane 1988). Influenza A viruses of the subtypes H5 and H7 caused massive epidemics in recent years in European, American and Asian countries (Swayne and Halvorson 2003). Further surveillance studies on wild birds, particularly waterfowl, are needed to elucidate the means by which the disease spreads and the methods of entry into farmed poultry.

The presence of *Chlamydia* spp. or chlamydial antigen in internal components of the eggs could not be established in this study. This negative result could potentially be a sampling error. It is known that the Canada goose itself, and occasionally its eggs, may be infected with this zoonotic agent (Wilt et al. 1972; Kaleta and Taday 2003). No data are currently available on chlamydial infections in humans who stayed for some time in the habitat of the geese. Further studies are needed to corroborate this result.

The egg-drop syndrome virus and its antibodies have been shown to have a wide host range (Schloer 1980; Kaleta et al. 2003). It appears that this adenovirus is in most instances of little, if any, pathogenic significance for geese (Bolte et al. 1997). However, this virus is stable outside the hosts and can induce severe inflammation of the salpinx of chickens, resulting in the formation of thin-shelled or even shell-less eggs. This problem can be prevented by individual vaccination of chickens with an inactivated vaccine. However, prevention the introduction of this virus from free-living waterfowl is considered a better approach (McFerran and Adair 2003).

The extensive studies on the isolation and species designation of aerobic bacteria yielded only a few isolates. Some of these could be correlated with embryo lesions. The isolation attempts for *Salmonella* spp. and *Cambylobacter* spp. were not successful. Glünder (1995) points out that these bacteria may account for embryo mortality and—more significantly—infection and enteric disease in humans.

During both study periods, a large number of eggs contained dead embryos. The exact reason(s) were not determined as far as infectious agents are concerned. At least some embryo mortality might be due to interrupted incubation by the parents. It is well known that disturbance by visitors forces the Canada goose to abandon its nest. Prolonged failure of incubation results finally in the death of the developing embryo (Brown 1988).

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