Characterization of avipoxviruses from wild birds in Norway

Simon Chioma Weli, Malachy Ifeanyi Okeke, Morten Tryland, Øivind Nilssen, Terje Traavik

Abstract

Avipoxviruses from different geographic regions of the world have been characterized to study their genetic and biological properties, but so far, no such work has been performed on Norwegian isolates. Lesions suggestive of avian pox, found on a Norwegian wild sparrow (*Passer domesticus*) and wood pigeon (*Palumbus palumbus*), were obtained in 1972 and 1996, respectively. Histologically, these lesions were demonstrated to be characteristic of poxvirus infections and the poxvirus was observed using an electron microscope. The resulting viruses were propagated in chicken embryo fibroblast cells. Restriction fragment length polymorphism of genomes from 2 Norwegian isolates and fowl pox vaccine strain, generated by *Bam*HI, revealed a high degree of heterogeneity among the isolates. The profiles of avipoxviruses isolated from wild birds were clearly distinct from each other and also to the fowl poxvirus strain. Furthermore, chickens experimentally infected with pigeon poxvirus had higher antibody titers and extensive lesions compared to other isolates. This may suggest that pigeon poxvirus is more virulent than the other isolates.

Résumé

*Les avipoxvirus de différentes régions géographiques du mode ont été caractérisés dans le but d'étudier leurs propriétés génétiques et biologiques, mais jusqu'à ce jour ce genre d'étude n'a pas été effectuée sur des isolats norvégiens. Des lésions suggestives d'infection par des poxvirus aviaires ont été trouvées en Norvège chez un moineau domestique (*Passer domesticus*) et un pigeon ramier (*Palumbus palumbus*) obtenu respectivement en 1972 et 1996. Les lésions histologiques étaient caractéristiques de celles des infections par les poxvirus et des poxvirus ont été observés par microscopie électronique. Les virus isolés ont été propagés sur des fibroblastes d'embryon de poulet. L'analyse du polymorphisme des fragments obtenus avec l'enzyme de restriction Bam*HI* des 2 isolats norvégiens et de la souche vaccinale du poxvirus du poulet a révélé un degré élevé d'hétérogénéité parmi les isolats. Les profils des avipoxvirus isolés des oiseaux sauvages étaient clairement distincts l'un de l'autre ainsi que de la souche vaccinale. De plus, des poulets infectés expérimentalement avec le poxvirus du pigeon avaient des titres en anticorps plus élevés et des lésions plus extensives que les poulets infectés avec les autres isolats. Ceci laisserait à penser que le poxvirus du pigeon est plus virulent que les autres isolats.*

(Traduit par Docteur Serge Messier)

Introduction

Members of the genus *Avipoxvirus* include several pathogens of avian importance (1) that replicate in vertebrate cells. Avipoxviruses have large genomes, 266 kilobases (kb) for attenuated strain FP9 and 288 kb for the United States Department of Agriculture challenge strain (2,3). Poxvirus infections have been documented in more than 200 species of wild and domestic birds (4). The disease produces lesions on the skin, mouth, esophagus, and trachea, although viruses may be found in other organs of affected birds, resulting in substantial economic losses in commercial poultry (5). However, there is no unified viewpoint yet, as to the exact number of species, strains, or variants that exist within the genus *Avipoxvirus* (4). Furthermore, information on genomic characterization, as well as pathogenicity and effects of avipoxvirus isolates from wild birds on commercial chickens, is limited.

Poxviruses have been used for years as vectors for the expression of foreign genes and the construction of recombinant vaccines (6). Accordingly, the orthopoxviruses, to which the smallpox virus and vaccinia virus belongs, are the best characterized (1). Recently, there has been renewed interest in the biology of viruses within the genus *Avipoxvirus*. This was stimulated by the fact that these viruses can be engineered to express foreign genes without undergoing a complete replication in mammalian cells (7).

Poxviruses from different geographic regions of the world have been characterized based on their genetic and biological properties (8–10). No such study has been performed on virus isolates from Norway. Disease caused by avipoxvirus had never been diagnosed in Norway until 1969 when the first cases were diagnosed in wild birds. In 1972, pox-like lesions were reported in different species of wild birds (11) and wild birds with poxvirus-like lesions are still being

Department of Microbiology and Virology (Weli, Okeke, Tryland, Traavik); Department of Medical Genetics (Nilssen), University of Tromsø, Brevika, N-9037 Tromsø, Norway; Norwegian Institute of Gene Ecology, Tromsø Science Park, N-9291, Tromsø, Norway (Traavik); and the Department of Arctic Veterinary Medicine, The Norwegian School of Veterinary Science, N-9292 Tromsø, Norway (Tryland).

Address all correspondence and reprint requests to Dr. Simon Chioma Weli; telephone: +47 77 64 46 61; fax: +47 77 64 53 50; e-mail: simonw@fagmed.uit.no

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observed. Despite these observations, no attempts were made to characterize the pathogen responsible.

In this study, we describe a comparative analysis of the genetic and biological characteristics of 2 avipoxviruses isolated from wild birds in Norway and a fowl poxvirus vaccine strain, in order to determine their relationships to each other and to the fowl poxvirus vaccine strain.

Materials and methods

Virus and cell lines

Two samples from wild birds with clinical signs of pox-like lesions were used as inocula for virus isolation. The 1st sample identified as sparrow poxvirus (SPV–A468), was collected in 1972 in Kristiansand, Norway, during an epizootic outbreak in a population of different wild bird species and brought to the National Veterinary Institute, Oslo, Norway. Histological examination revealed proliferation in the epithelial cells, while homogenates of the skin proliferation produced pocks when inoculated onto the chorioallantoic membrane (CAM) of chick embryos (11). The 2nd sample, identified as wood pigeon poxvirus (PPV–B7), was obtained in 1996 from Judaberg, Norway and brought to the Department of Virology, University of Tromsø, Norway, for analysis. Skin lesions were excised and grounded with sterile alundum (60 Mesh Norton Alundum "RR;" Fisher Scientific Company, Pittsburgh, Pennsylvania, USA) in Hank's balanced salt solution containing 1000 U/mL penicillin, 1 mg/mL streptomycin, and 2.5 μg/mL amphotericin B.l (Life Technologies, Gaithersburg, Maryland, USA). The suspension was incubated at 37°C for 1 h and clarified by low-speed centrifugation at 1500 × *g* for 5 min. Approximately 100 μL of the supernatant was inoculated onto chicken embryo fibroblast (CEF) cells (American Type Culture Collection ATCC, Rockville, Maryland, USA) and passaged 3 times. Plaque assay was performed. The isolates were identified as poxvirus by electron microscopy (EM). In addition, a fowl poxvirus vaccine strain (FPV–VR250) obtained from (ATCC) was used as a reference strain for comparison. All viruses were propagated in CEF cells in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 5% tryptose broth, and 5% fetal bovine serum.

Electron microscopy of avipoxvirus-infected cells

Negative staining — Confluent CEF cells were separately infected with fowl poxvirus, sparrow poxvirus, and pigeon poxvirus at multiplicity of infection (MOI) 25. At 48 h postinfection (pi) the medium was removed, cells scraped from one culture flask and resuspended in 9 mL sterile distilled water. The cells were subjected to 3 cycles of freezing and thawing, and cell debris removed by low-speed centrifugation at 1000 × *g* for 5 min at room temperature (Sorvall; Dupont NEN Research Products, Boston, Massachusetts, USA). The supernatant fluid was centrifuged at 40 000 × *g* for 20 min at 4°C (Beckman Instruments, Palo Alto, California, USA). The pelleted viruses were then resuspended, negatively stained with 1% phosphotungstic acid (pH 6.2) and examined in an electron microscope (JEOL JEM–1010).

Transmission electron microscopy (TEM)

Confluent CEF cells grown in 8-well culture plate (Nalga Nunc International, Naperville, Illinois, USA) were infected with avipoxviruses at MOI 5 and incubated at 37°C. At 72 h pi the cultures were washed 3 times with culture medium, fixed in 1% glutaraldehyde at 4°C for 1 h, washed in medium, and post-fixed in 1% osmium tetroxide for 1 h at 4°C. After 1 wash in medium, cultures were dehydrated in graded ethanol, and embedded flat in epoxy resins. Ultra-thin sections were cut parallel to the surface of the dish with a diamond knife. Uranyl acetate and lead citrate were added as a contrast agent and the sections were examined using an electron microscope (JEOL JEM–1010).

Isolation, restriction enzyme digestion, and gel electrophoresis of viral DNA

Viral DNA was extracted from infected cells as described by Esposito et al (12). Restriction endonucleases (New England Biolabs, Beverly, Massachusetts, USA) were obtained and used as specified by the manufacturer. The DNA fragments were separated in 0.5% agarose gel (SeaKem LE Agarose; BioWhittaker Molecular Application, Rockland, Maine, USA) (20 × 20 cm) containing 1× TAE (0.4 M Tris-acetate, 0.02 M ethylenediaminetetracetic acid [EDTA] pH 8, 11.4 g glacial acetic acid) at 25 volts for 22 h. Gels were stained with 0.5 μg/mL ethidium bromide at room temperature for 15 min and destained for 45 min at 4°C in distilled water. The gel was then photographed (Bio-RAD Gel Doc 2000 trans-illuminator; BIO-RAD, Hercules, California, USA).

Viral pathogenicity in chickens

Twenty 6-week-old specific pathogen-free chickens obtained from Senja, Norway, were used. Five chickens were inoculated with each CEF cell-adapted purified poxvirus. Each chicken received 40 μL of virus containing 1.2×10^5 PFU/mL subcutaneously in the left wing web. Five chickens were kept as uninfected controls. The chickens were housed in separate cages in different isolation units under negative pressure in the Animal Laboratory Facility, University of Tromsø, Norway, under the care of Norwegian Association of Laboratory Animal Science (NALAS) certified animal care staff. The study was granted ethics approval by the ethics committee of the University of Tromsø, Norway. Blood samples were taken from the wing veins of each chicken before inoculation and at 3-day intervals pi. All chickens were examined daily for development and regression of cutaneous pox lesions, characterized by the appearance of nodular lesions at the site of poxvirus inoculation.

Antigen preparation

The CEF cell monolayer grown in 4, 175 cm^2 culture flasks were infected with fowl poxvirus, sparrow poxvirus, and pigeon poxvirus. At 2 h pi, the inoculum was removed and the cells were washed twice with PBS. The cell layers were covered with culture medium containing 2% fetal bovine serum until CPE was observed, 4 to 5 d pi. Cells were harvested and pelleted by centrifugation at 1500 × *g* for 10 min at 4°C. The supernatant was discarded and the cell pellet was washed with isotonic buffer (10 mM Tris, pH 8.0, 10 mM NaCl, 5 mM EDTA). The cells were then lysed in 10 mL hypotonic buffer

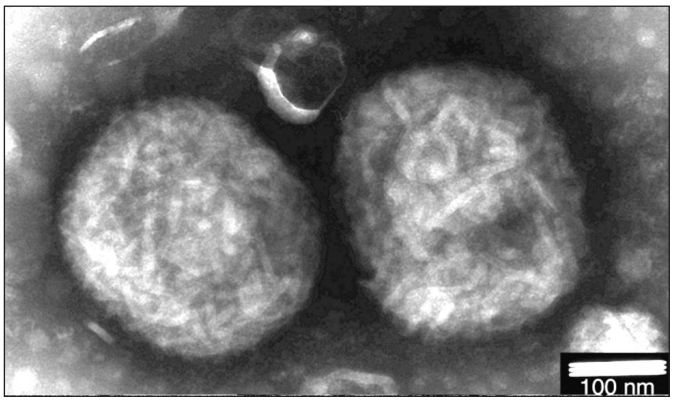

Figure 1. Electron micrograph of negatively stained poxvirus in chicken embryo fibroblast (CEF) cells.

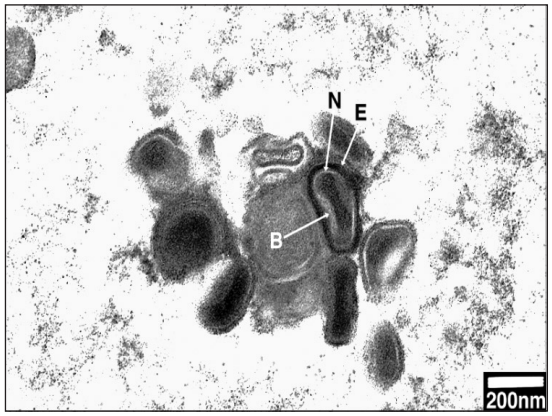

Figure 2a. Electron micrograph of ultra-thin section of mature pigeon poxvirus. Arrows indicate brick-shaped virus with an electron-dense biconcave nucleocapsid core (N), an intermediate coat enclosing 2 lateral bodies (B) and an outer lipoprotein coat (E).

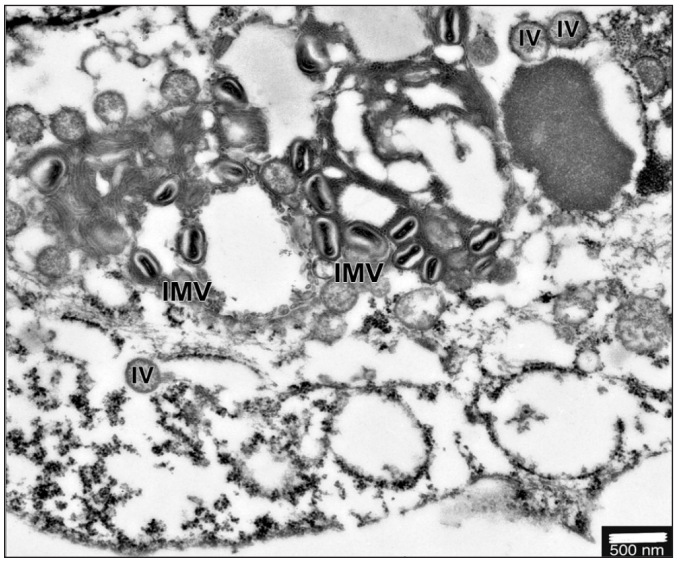

Figure 2b. Electron micrograph of ultra-thin section, showing intracellular mature virus (IMV) and immature virus (IV) in chicken embryo fibroblast (CEF) cells.

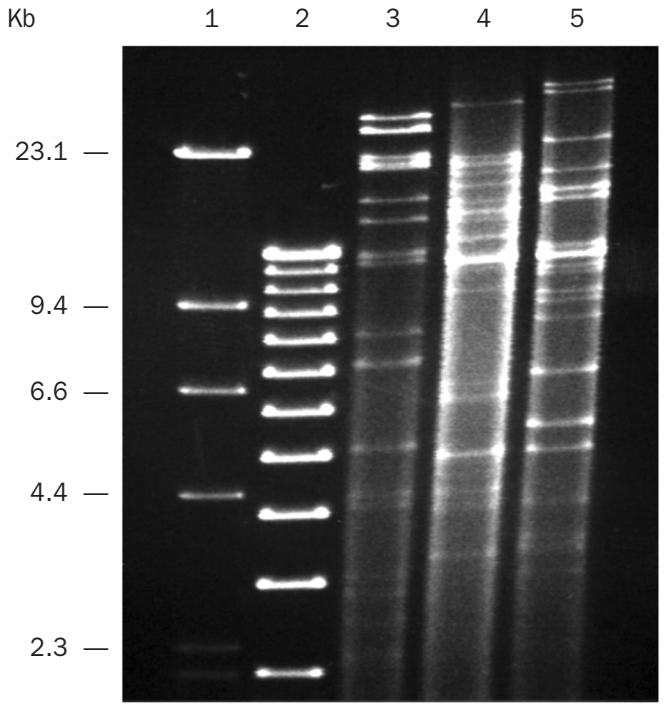

Figure 3. Agarose gel electrophoresis of DNA of avipoxviruses after cleavage with *Bam*HI. Lane 3, fowl poxvirus vaccine strain; lane 4, sparrow poxvirus; lane 5, pigeon poxvirus; lanes 1 and 2, lambda DNA-*Hind*III/ΦX-174 DNA-*Hae* III and 1 kb markers, respectively.

(10 mM Tris, pH 8.0, 10 mM KCl, 5 mM EDTA) containing 0.1% Trition X-100 and 0.025% β-mercaptoethanol. Nucleic and cell debris were removed by centrifugation at 450 × *g* for 5 min at 4°C. The supernatant was used as the antigen for an enzyme-linked immunosorbent assay (ELISA).

Enzyme linked immunosorbent assay

Immulon Maxi sorp 96-well microtiter plates (NUNC A/S, Roskilde, Denmark) were coated with 1 μg/mL of the different avipoxvirus antigens in 100 μL of carbonate-bicarbonate buffer (pH 9.6) per well and incubated overnight at 4°C. Concentration of the antigen was determined with a protein reagent kit (Bio-Rad). The coated wells were washed once with 150 μL/well of wash solution (0.29 M NaCl, 0.05% Tween 20). Nonspecific binding sites were blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7.4, for 1 h at 37°C. After 1 wash, test sera from chickens inoculated with poxviruses were added to each well. The plates were incubated for 2 h at 37°C. After washing 3 times with wash solution, plates were incubated for 1 h at 37°C with a secondary antibody, rabbit anti-chicken immunoglobulin (Ig) G conjugated with horseradish peroxidase (HRP) (Sigma, St. Louis, Maryland, USA), diluted 1:3000 in PBS containing 1% BSA. The wells were washed twice and the bound antibodies were detected with a TMB-peroxidase kit (Kairkegaard and Perry Laboratories, Gaithersberg, Maryland, USA) according to the manufacturer's instruction. The reaction was stopped using 1 M phosphoric acid and the absorbance at 450 nm was determined using an ELISA plate reader (Dynatech Laboratories, Chantilly, Virginia, USA).

Table I. Sizes of DNA fragments of different avipoxviruses after digestion with *Bam*HI

Fowl poxvirus		Sparrow poxvirus		Pigeon poxvirus	
DNA fragment	kb	DNA fragment	kb	DNA fragment	kb
A	39	A	44	A	49
B	28	B	22	B	47
C	27	C	21	C	23
D	23	D	18.9	D	18.7
E	22	E	18.8	E	16.8
F	21	F	18.6	F	16.2
G	20	G	16.4	G	12.2
H	18.5	H	16	H	11.2
I	15.9	I	14.1	I	10.9
J	12.9	J	11.2	J	10.4
K	12	K	11.0	K	9.5
L	9.9	L	10.4	L	9.0
M	8.8	M	8.5	M	8.4
N	7.6	N	6.0	N	6.8
O	7.4	—	4.2	O	5.6
P	5.3	—	—	P	4.9
Q	4.1	—	—	Q	4.0
Total fragments	17	—	15	—	17
Total size	282.4	—	241.1	—	263.6

kb — kilobases

Results

To characterize the agent responsible for the lesions observed in wild birds, homogenates of the skin proliferation from the bird (sparrow) with pox-like lesions were inoculated onto the CAM (11). By 7 d pi morphological changes consisting of individual pocks in the CAM were seen. Histological examination of the pock revealed massive proliferation of the ectodermal epithelium and edema in the interepithelial connective tissue (11). The sparrow poxvirus and pigeon poxvirus isolates were inoculated onto CEF cell cultures. Plaque assay of the wild bird isolates revealed significant differences in the rate of the plaque development and sizes of plaques. The sparrow poxvirus produced large (2 to 3 mm), defined plaques in 4 d, but by day 6 few diffuse plaques were observed for pigeon poxvirus. This was followed by the demonstration of poxvirus using negative-staining electron microscopy (Figure 1). Ultra-thin sections of infected cells showed abundant oval and brick-shaped poxviruses, some of which had the conspicuous, dumbbell-shaped core (Figures 2a and 2b). Immature virus was identified in the cytoplasm of the infected cells (Figure 2b).

Since 2 of the viruses originated in different species of wild birds, a genetic analysis was performed to determine their relationship to each other and to the fowl poxvirus vaccine strain. A comparison of the restriction fragments length polymorphism (RFLP) profiles generated by *Bam*HI digestion of the genomes revealed a lack of comigrating fragments between the 2 Norwegian isolates and the fowl poxvirus (Figure 3). *Bam*HI DNA digest of fowl poxvirus, sparrow poxvirus, and pigeon poxvirus produced 17, 15, and 17 fragments, respectively, with sizes ranging from 4.0 to 49.0 kb (Table I). The total genomic size was estimated to be approximately 282.4 kb (fowl poxvirus), 241.1 kb (sparrow poxvirus), and 263.6 kb (pigeon poxvirus), respectively. The *Bam*HI digestion of the pigeon poxvirus DNA produced 2 large fragments of 49.0 and 47 kb, while the sparrow poxvirus DNA produced a large fragment of 44 kb. In contrast, 2 large fragments of 39 and 29 kb were observed for fowl poxvirus DNA. A number of small, poorly resolved fragments were not taken into account while calculating the total size of the genomes.

To further characterize the Norwegian wild bird avipoxviruses isolates, a pathogenicity study was performed to determine whether the wild bird isolates could pose a threat to commercial chickens. Chickens inoculated with fowl poxvirus strain developed localized lesions at the site of inoculation after 3 d, and regressed after 3 wk, while those inoculated with sparrow poxvirus did not have any lesions at the site of inoculation (Table II). On the other hand, the inoculation of pigeon poxvirus resulted in severe lesions at the site of inoculation that persisted for more than 3 wk. In addition, secondary lesions were observed close to the lower and upper beak of the chickens inoculated with fowl poxvirus strain and pigeon poxvirus. The antibody titers determined by ELISA revealed that all avipoxvirus inoculated chickens showed high antibody responses against avipoxvirus antigens (Table III). Pigeon poxvirus inoculated chickens had the highest antibody responses on day 12, followed by fowl poxvirus and sparrow poxvirus inoculated chickens, respectively. Beginning on day 16, the responses decreased until the end of the experiment.

Discussion

This study was designed to characterize avipoxviruses obtained from wild birds in Norway and to compare them with a fowl poxvirus strain that infects commercial chickens. Since such pathogens may

Table II. Pathogenicity of avipoxvirus isolates in chickens

Virus isolate	Type of lesions[a] at 1 wk	Lesions at 2 wk	Lesions at 3 wk
Fowl poxvirus (VR250)	+	++	++S
Sparrow poxvirus (A468)	–	–	–
Pigeon poxvirus (PPV-B7)	++	++	++S

[a] Absence of lesions at site of inoculation; + mild localized lesion/nodule at the site of inoculation; ++ extensive proliferative lesions at the site of inoculation; S secondary lesions at other sites

Table III. Antibody responses of chickens inoculated with avipoxviruses measured by enzyme linked immunosorbent assay (ELISA)

	Group		
Time	VR-250	SPV-A468	PPV-B7
Day 0 (preinoculation)	1.020 ± 0.099	1.051 ± 0.118	1.557 ± 0.448
Day 3 (postinoculation)	1.464 ± 0.405	1.138 ± 0.275	2.323 ± 0.193
Day 5 (postinoculation)	1.648 ± 0.276	1.165 ± 0.288	2.416 ± 0.177
Day 8 (postinoculation)	2.192 ± 0.327	1.596 ± 0.136	2.694 ± 0.414
Day 10 (postinoculation)	2.295 ± 0.374	1.668 ± 0.227	2.804 ± 0.394
Day 12 (postinoculation)	2.559 ± 0.532	1.796 ± 0.197	3.412 ± 0.312
Day 16 (postinoculation)	2.149 ± 0.153	1.611 ± 0.248	3.055 ± 0.267
Day 23 (postinoculation)	2.122 ± 0.279	1.589 ± 0.225	3.022 ± 0.174
Day 30 (postinoculation)	2.002 ± 0.095	1.443 ± 0.344	2.734 ± 0.319

Mean ± standard deviation (s) of absorbance at 450 nm from 5 chickens in each group.
VR-250 — fowl poxvirus; SPV-A468 — sparrow poxvirus; PPV-B7 — pigeon poxvirus

play a role in limiting the population of commercial chickens (13,14) a pathogenicity test in chickens was also performed.

The lesions observed on birds and the negative-stained preparations from the Norwegian sparrow and wood pigeon confirmed the presence of poxvirus in wild birds. Moreover, electron microscopy of thin sections of virus infected in CEF cells indicated that early and late stages of viral assembly (crescent and spherical forms) occurred, and matured virus with the characteristic dumbbell-shape was observed. These findings were consistent with other reports of cases of poxvirus in birds (5,15–17).

Restriction enzyme digestion of DNA had been previously used to characterize poxviruses. Such analysis revealed that members of the genus *Orthopoxvirus* have considerable similarity (18,19), while members of the genus *Parapoxvirus* contain DNA with extensive heterogeneity (20). The *Bam*HI DNA digest of fowl poxvirus, sparrow poxvirus, and pigeon poxvirus revealed genomic differences between the 2 Norwegian isolates and the fowl poxvirus strain, indicating that genetically distinct poxviruses do exist among Norwegian wild birds. Indeed, all the isolates can be clearly distinguished by the presence or absence of multiple DNA fragments. The sum of the fragment sizes gave an estimated genome size of 282.4 kb, 241.1 kb, and 263.6 kb, respectively, for fowl, sparrow, and pigeon poxviruses. However, difficulties in making accurate size measurements of large DNA fragments means that the genome size of the fowl poxvirus should probably be regarded as approximately 288 kb, which is in agreement with the size reported by Alfonso et al (2). The interspecies genetic heterogeneity observed here with regards to the wild bird isolates was not surprising when compared with previous reports (10,21). What was unique was the apparent genetic heterogeneity between the fowl poxvirus and the Norwegian pigeon poxvirus isolate, since a previous report had shown that pigeon poxvirus is closely related to fowl poxvirus (21). The Norwegian wood pigeon is considered to be a migratory bird (22). Whether this particular wood pigeon was infected by a distinct poxvirus from a different geographic region remains unclear.

Although the 2 poxvirus isolates originated from wild birds in Norway, it is not clear whether they pose a threat to commercial chickens. In commercial chickens, malaria (13) and poxvirus infections (14) have been considered to be important factors responsible for substantial economic losses. But little information is available about the direct effects of the wild bird pathogens on the survival of commercial chickens. Susceptibility of commercial chickens to fowl poxvirus and pigeon poxvirus was studied. Chickens infected with pigeon poxvirus had higher antibody titers and more extensive lesions compared to other isolates. This may suggest that the pigeon poxvirus was more virulent than the other isolates. But whether this virus effect would cause severe loss of chickens in case of an epidemic is not known, since none of the chickens died during the study.

In this study, genetic and biological characterization of Norwegian avipoxvirus isolates was performed in order to determine their relationship to each other and to the fowl poxvirus strain. Although limited number of isolates and strains were studied, the obvious genetic differences among the 3 viruses confirm that this genus is unique in the poxvirus family and would suggest that more information on avipoxviruses is needed.

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