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ABSTRACT: Two hundred and twenty one blood samples representing eight different rodent species and the common shrew (*Sorex araneus*), collected in Norway between 1993 and 1995, were examined for anti-orthopoxvirus antibodies by a competition enzyme linked imunnosorbent assay (ELISA) and, when possible, an indirect immunofluorescence assay. The serological results indicated that the bank vole (*Clethrionomys glareolus*), woodmouse (*Apodemus sylvaticus*) and Norway lemming (*Lemmus lemmus*) may be reservoir species for orthopoxviruses in Norway, with antibody prevalences of 17 (12/69), 30 (24/81) and 56% (19/34), respectively. Orthopoxvirus infection in lemmings has not been reported previously. On some other small rodent species such as field voles (*Microtus agrestis*), common rats (*Rattus norvegicus*), and common shrews, seropositive individuals were detected. However, the total number of tested animals was low, and the role of these species in the epidemiology of orthopoxvirus infections remains unclear. Attempts to isolate orthopoxviruses from these small mammals failed, although orthopoxvirus specific DNA sequences were detected previously in the same animals by the polymerase chain reaction (PCR). The serological results were compared with and discussed in the context of the occurrence of orthopoxvirus-specific DNA sequences, and it is concluded that orthopoxviruses are widely distributed among wildlife in Norway.

Key words: Competition enzyme-linked immunosorbent assay, cowpox virus, ELISA, orthopoxvirus, rodents, shrews, wildlife reservoir.

INTRODUCTION

Little is known about the occurrence of orthopoxviruses in wildlife species. The genus *orthopoxvirus* within the family Poxviridae consists of several species causing diseases in a wide range of animal species and in humans (Fenner, 1996). The worldwide epidemics of smallpox caused by variola virus led to intensive investigation on the diagnostics of orthopoxviruses (Fenner et al., 1989a), and new hosts and virus species have been detected. Raccoonpox virus (Thomas et al., 1975), volepox virus (Regnery, 1987) and skunkpox virus (Fenner, 1996) were discovered recently as new orthopoxvirus species in North America. In western Europe, the number of clinical orthopoxvirus infections in man, domestic cats and zoo animals have been reported increasingly, and virus isolates from such cases have been characterized most often as variants of cowpox virus (Thomsett et al., 1978; Bennett et al., 1990; Bomhard et al., 1992). The host range of cowpox virus is wide, and includes humans, cattle, domestic cats and dogs, several rodent species, and several zoo species (large felines, elephants, okapis, rhinoceroses, and anteaters) (Marennikova et al., 1984; Fenner et al., 1993). Isolation of cowpox virus from rodents has only been successful from gerbils (*Rhombomys opimus*) and yellow susliks (*Citellus fulvus*) in Turkmenia (Marennikova et al., 1978), and from red-tailed Libyan jird (*Meriones libyans*) in Georgia and root vole (*Microtus oeconomus*) on the Kola Peninsula in Russia (Pilaski and Jacoby, 1993). However, serological surveys of small wild rodents have revealed that orthopoxviruses circu-

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late in several rodent species in Turkmenia (Marennikova, 1979), Great Britain (Kaplan et al., 1980; Crouch et al., 1995), Belgium (Boulanger et al., 1996) and Germany (Pilaski and Jacoby, 1993), which has contributed to the acceptance of their role as the reservoir of cowpox virus. Recently, orthopoxvirus-specific antibodies also have been detected in red foxes (*Vulpes vulpes*) and wild boars (*Sus scrofa*) in Germany (Henning et al., 1995; Mayr et al., 1995).

Cowpox was known previously as a disease in milking cows. There were 5,223 clinical cases of "cowpox" infections in milking cows reported in Norway in 1928. At that time such infections were considered one of the most important diseases in cattle, causing teat ulcers leading to mastitis and economical losses (Holth, 1930). However, some of these reported cowpox cases might have been vaccinia mammilitis, milkers nodule's or bovine herpes mammilitis. Virus was believed to be transmitted to cattle from humans vaccinated against smallpox with cowpox virus and later vaccinia virus which were propagated in calves (Holth, 1930). Transmission of virus from vaccinees to cows, from cow to cow, and from cows back to humans also are reported by others (Gibbs et al., 1973; Kaplan, 1989). Apparently, cowpox was not recognized with certainty in Norway until the autumn of 1994, when one feline and one human case appeared (Tryland et al., 1996; Myrmel et al., 1997). There are no reports of clinical orthopoxvirus infections in rodents from Norway, but orthopoxvirus DNA has been detected in several wild rodent species and in common shrews by the polymerase chain reaction (PCR) and southern blot analysis (T. Sandvik, unpublished data).

Poxviruses have been engineered for use as recombinant, live vaccine vectors intended for use in man, domestic animals and wildlife (Yilma, 1994; Perkus et al., 1995). A recombinant vaccinia-rabies virus vaccine has been used to vaccinate the red fox against rabies in Belgium and France since 1988 (Pastoret and Brochier, 1996) and field trials using live vaccinia rabies G recombinant already have been conducted in the USA and are contemplated for Canada (Artois et al., 1990; Fletcher et al., 1990; Hable et al., 1992). The possible risks associated with the use of recombinant poxvirus vaccines (Kaplan, 1989), such as potential recombination (Ball, 1987; Gershon et al., 1988) and the possibility of vaccinia virus establishing reservoirs in nature and causing infections in humans as seem to be the case for the vaccinia subspecies buffalopox virus, a zoonotic agent transmitted from milking buffalo and diary cattle to humans (Dumbell and Richardson, 1993), makes knowledge about the occurrence and ecology of related, naturally distributed orthopoxviruses important.

The aim of this study was to design an orthopoxvirus-specific competition enzyme linked imunnosorbent assay (ELISA) for screening of sera from different rodent species and the common shrew from different parts of the country. The results are compared with immunofluorescence (IF) and polymerase chain reaction (PCR) data.

MATERIALS AND METHODS

Serum Samples

Two hundred and twenty one serum samples were collected from bank voles (*Clethrionomys glareolus*), northern red-backed voles (*Clethrionomys rutilus*), grey-sided voles (*Clethrionomys rufocanus*), wood mice (*Apodemus sylvaticus*), root voles, field voles (*Microtus agrestis*), common rats (*Rattus norvegicus*), Norway lemmings (*Lemmus lemmus*), and common shrews (*Sorex araneus*) (Table 1). The trapping locations were chosen to obtain blood samples from the characteristic species in different biotopes and ecosystems. Exact locations are shown in Figure 1. The animals were caught alive in traps (Ugglan Special, Grahn AB, Sweden), in spring and autumn 1993–95. The traps were baited with seeds and potato or apple, and placed close to holes in the ground made by the small mammals. The traps were checked several times each 24 hr period during the 4 to 5 day trapping period at each location and season. At the most, 250 traps were used, concentrated in small areas only 100 to 200 m in diameter. Blood samples were collected by syringe and needle, both previously flushed with

TABLE 1.   Regional distribution of small rodents and common shrews (*Sorex araneus*) with antiorthopoxvirus antibodies in Norway.

Region Mammal species	Competition-ELISA: % inhibition		IF[a]	PCR[b]
	>80%	>90%		
1. Masi, Finnmark[c]				
Northern red-backed vole[d]	1/9 (11)[e]	0/9 (0)	0/9 (0)	3/9 (33)
Grey-sided vole	0/9 (0)	0/9 (0)	0/9 (0)	2/9 (22)
Root vole	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)
2. Bygstad, Sogn & Fjordane				
Field vole	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)
Common shrew	1/1 (100)	1/1 (100)	—	0/1 (0)
3. Austrheim, Hordaland				
Wood mouse	8/18 (44)	5/18 (28)	1/18 (6)	3/18 (17)
Field vole	1/1 (100)	1/1 (100)	1/1 (100)	0/1 (0)
Common shrew	1/1 (100)	0/1 (0)	—	0/1 (0)
4. Kalandsvatnet, Hordaland				
Bank vole	3/4 (75)	1/4 (25)	4/4 (100)	2/4 (50)
Wood mouse	25/55 (45)	10/55 (18)	12/55 (22)	13/55 (24)
Common shrew	2/4 (50)	1/4 (25)	—	1/4 (25)
5. Hardangervidda (National Park)				
Grey-sided vole	2/4 (50)	0/4 (0)	0/4 (0)	1/2 (50)
Norway lemming	2/14 (14)	1/14 (7)	—	0/14 (0)
6. Søgne, Vest-Agder				
Bank vole	9/9 (100)	8/9 (89)	8/9 (89)	0/9 (0)
Wood mouse	4/4 (100)	4/4 (100)	0/4 (0)	0/4 (0)
7. Kongsvinger, Hedmark				
Bank vole	2/55 (4)	2/55 (4)	0/55 (0)	7/55 (13)
Common shrew	1/1 (100)	1/1 (100)	—	0/1
8. Valdres, Oppland				
Norway lemming	20/20 (100)	18/20 (90)	—	NT
9. Miscellaneous[f]				
Bank vole	1/1 (100)	1/1 (100)	0/1 (0)	NT
Wood mouse	4/4 (100)	4/4 (100)	0/4 (0)	NT
Common rat	2/2 (100)	2/2 (100)	0/2 (0)	NT

[a] Immunfluorescence. A titer >10 was considered positive. Results not comparable (—).

[b] Polymerase chain reaction results from the same animals (T. Sandvik, unpublished data); NT, not tested.

[c] Number and names refer to Fig. 1 (map).

[d] See text for scientific names of mammal species.

[e] Number of seropositive individuals/number of tested individuals (percentage of seropositive individuals).

[f] Miscellaneous refers to different sites in Oslo and the island Skåtøy in Telemark.

heparin (Heparin® 5000 IE/ml; Nycomed Pharma AS Oslo, Norway) to avoid clotting, by heart puncture under ether anaesthesia. From adult mice the range of blood collected was 0.3 to 1.5 ml (average 0.6 ml) and from shrews, when not dead in the traps, the range of blood available was 0.1 ml to 0.6 ml (average 0.3 ml). The animals died while asleep during the sampling, and the carcasses were stored on dry ice for PCR analysis. Blood was transferred from the syringe to microcentrifuge tubes (Costar Snap Cap; Corning Costar Europe, Badhoevedorp, The Netherlands) and serum separated in a Costar Minicentrifuge (Model 10MVSS). The serumsamples were kept on dry ice, and later stored at −20 C until tested.

Control sera

Vaccinia virus, strain Western Reserve, [American Type Culture Collection (ATCC), Rockville, Maryland, USA; number VR119] was
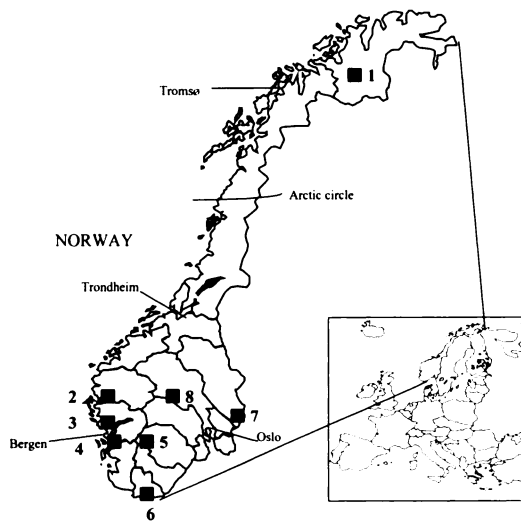
FIGURE 1. Sampling locations for orthopoxvirus testing of eight different rodents species and the common shrew in Norway. Local names and county (map coordinates for the center of each location): 1. Masi, Finnmark (69°25'N, 23°30'E); 2. Bygstad, Sogn og Fjordane (61°22'N, 5°40'E); 3. Austrheim, Hordaland (60°47'N, 4°55'E); 4. Kalandsvatnet, Hordaland (60°17'N, 05°25'E); 5. Hardangervidda National Park (60°16'N, 07°35'E); 6. Søgne, Vest-Agder (58°05'N, 07°45'E); 7. Kongsvinger, Hedmark (60°15'N, 12°15'E); and 8. Valdres, Oppland (61°00'N, 09°00'E).

propagated in Vero cell monolayers (ATCC number CCL81), using Eagles Minimum Essential Medium (GibcoBRL, Life Technologies inc., Gaithersburg, Maryland, USA) supplemented with 5% bovine calf serum, L-glutamine (2 mmol/litre) and antibiotics (Benzylpenicillin 1.04×10^5 units/l and Streptomycin sulphate 7.87×10^4 units/l; Sigma Chemical Co., St. Louis, Missouri, USA). The cultures were incubated at 37 C in a 100% humidity and 5% CO_2 atmosphere. Infected cells were frozen when 80 to 90% of the cells showed cytopathic effect (CPE), 3 to 4 days post infection. Virus was purified by a standard method (Joklik, 1962) with the following slight modifications. After centrifugation through 40% sucrose, the pellet was resuspended in TE-buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and loaded on a metrizamide-gradient (Nycomed Pharma AS, Oslo, Norway) with a refractive index range from 1.39 to 1.41, centrifuged in a Beckman SW50.1 rotor (Beckman Instruments Inc., Fullerton, California, USA) at 4 C and 20,000 × g for 18 hr. The virus bands in the gradients were collected by a peristaltic pump, dialyzed against phosphate buffered saline (PBS) and frozen at

−70 C until further use. Purified virus, approximately 7×10^8 plaque forming units (PFU) in PBS was mixed 1:14 with PBS containing 0.4% glutaraldehyde, placed on a shaker for 48 hr for inactivation, and subsequently checked for lack of CPE in Vero cell monolayers. The protein content of the inactivated virus suspension was measured by a BCA protein assay reagent kit (Pierce Chemical Company, Rockford, Illinois, USA) to 1,800 µg/ml.

A blood sample from a 3 kg female New Zealand White rabbit (Harlan UK Limited, Oxon, UK) was used as a negative control. The rabbit was subsequently immunized subcutaneously with 450 µg of vaccinia virus protein from the suspension of glutaraldehyde inactivated virus with Freunds complete adjuvant (1:1) in a total volume of 3 ml. Inactivated virus was used for immunization because of restricted facilities to deal with live virus. A total of five boosts of 200 µg vaccinia virus protein with Freunds incomplete adjuvant were given at 2 or 3 wk intervals, before the rabbit was bled under anaesthesia (Hypnorm® (fentanyl/fluanison) 0.1 ml/kg i.m.; Janssen Pharmaceutica BV, Beerse, Belgium) and killed. The hyperimmune serum was separated by centrifugation and stored at −20 C. Hyperimmune serum from the rabbit as well as from a bank vole and a domestic cat, both experimentally infected in other laboratory facilities with infectious cowpox virus by a single footpad and intradermal inoculation respectively, were used as positive control sera.

From the rabbit hyperimmune serum, the fraction of immunoglobulin G (IgG) was purified by affinity chromatography on a 5 ml protein-A column (HiTrap® affinity column, Pharmacia Biotech, Uppsala, Sweden) and a FPLC-system (LCC-501 Plus Core System, Pharmacia), according to the manufacturers instructions. Before use, the IgG solution was dialyzed against 0.1 M $NaHCO_3$ + 0.15 M NaCl, pH 8.5, and coupled to a biotin-N-hydroxysuccinimide ester according to the suppliers protocol (GibcoBRL, Life Technologies inc., Gaithersburg, Maryland, USA). Unbound biotin and salts were removed by dialyzing overnight against 5 l of PBS.

Competition ELISA

Microtiter plates (Nunc-Immuno® PolySorp, NUNC A/S, Roskilde, Denmark) were coated overnight on a shaker with 100 µl purified infectious vaccinia virus in 0.1 M Na-carbonate buffer, pH 9.5 at a final concentration of 4.5 µg protein/ml measured by the BCA protein assay reagent kit (Pierce). Infectious virus with unmodified antigen structures was considered to be immunologically most suited. After washing 5 times with PBS containing 0.05%

Tween 20 (PBS-T) in a Skatron Microwash II (Scatron Instruments AS, Lier, Norway) (repeated between each step in the competition-ELISA), the plates were blocked for 1 hr with 200 μl PBS containing 3% Tween 20. Serum samples (test sera and controls) were diluted 1:10 and 1:100 in PBS-T with biotinylated IgG (150 μg/ml, 1:100). The dilutions were carried out in duplicates in polystyrene microtiter plates (Nunc Microwell®, NUNC A/S, Roskilde, Denmark) not treated to enhance protein binding. Preimmune rabbit serum (1:100) was added to the dilution mixture to ensure that any non-specific effect of the presence of serum would contribute to the optical densities measured in all wells, and to minimize non-specific binding of orthopoxvirus specific antibodies. Vero cell protein (1 mg/ml, 1:100) was added to minimize possible binding of the biotinylated rabbit IgG to Vero cell protein in the antigen preparation. The serum dilutions were incubated for 2 hr in the polystyrene microtiter plates in order for immunocomplexes to be formed, before they were applied to the coated plates. After 1 hr incubation, streptavidin-peroxidase (POD) conjugate (Böhringer Mannheim, GmbH, Mannheim, Germany) was added in a 1:10,000 dilution in PBS-T. After 30 min of incubation, 1.5 mg/ml orthophenylenediamine (OPD; DAKO, Glostrup, Denmark) in citric acid phosphate buffer, pH 5.0, containing 0.6 μl 30% H_2O_2/mg OPD, was added as substrate, incubated for 10 min and the reaction stopped with 1M H_2SO_4. The plates were read in a spectrophotometer (Labsystems Multiskan Bichromatic type 348, LabSystems OY, Helsinki, Finland) at 492 nm. The 1:10 dilution of the test sera gave a better differentiation of the samples than the 1:100 dilution, and was chosen for further calculations. Percent reduction of the photometer extinction of the biotinylated rabbit IgG by the competing test sera was calculated by the formula: %-inhibition = [OD_{492} rabbit preimmune serum − OD_{492} test serum]/[OD_{492} rabbit preimmune serum − OD_{492} rabbit hyperimmune serum] × 100. By this calculation, the 1:10 dilution of the hyperimmune serum from the domestic cat infected with cowpox virus (positive control) gave an inhibition of 112% compared to the rabbit hyperimmune serum. Due to restricted volume, the hyperimmune serum from the cowpox-infected bank vole was only tested in 1:100 dilution, and showed an even stronger inhibition of the test than the cat serum in the same dilution.

To demonstrate that the ability of the sera to inhibit in the competition ELISA was IgG-specific, IgG from sera with inhibition levels above 90% from a bank vole, a wood mouse and a Norway lemming, and from the rabbit immu-

nized with inactivated vaccinia virus and a cat immunized with infectious cowpox virus (positive controls), was separated on a protein A column and a FPLC system as described above for rabbit-anti-vaccina virus IgG. Starting volume of serum varied between 0.5 and 1.0 ml. The separation gave different amounts of IgG from the different animal species, due to species-differences in the ability of IgG to bind to protein A (Lindmark et al., 1983). Rabbit and domestic cat IgG showed high affinity to protein A, bank vole and Norway lemming only moderate affinity, whereas wood mouse IgG demonstrated very restricted affinity to protein A. The IgG fractions were dialyzed against PBS and tested in the competition ELISA.

Immunofluorescence

Serum samples were serially diluted twofold in PBS from an initial dilution of 1:10 and the immunofluorescence assay was done as described by Crouch et al. (1995), modified to use a mixture of commercial 1:15 α antimouse and 1:25 α antirat fluorescein isothiocyanate (FITC)-conjugates (Sigma Chemical Co., St. Louis, Missouri, USA) to detect binding of rodent antibodies from the test sera. Specific intracytoplasmic fluorescence was detected in infected cells, and the IF-titer was taken as the resiprocal of the highest dilution where fluorescence could be detected. An IF titer of ≥10 was considered positive. Sera from experimentally infected bank voles were used as positive controls.

Virus isolation and PCR on cell cultures

Samples of lung, liver, spleen and kidney from four bank voles, six wood mice and one common shrew containing orthopoxvirus DNA sequences as detected by PCR and with inhibition values in the competition ELISA varying from 35 to 90% were cut in small pieces, macerated in PBS in microcentrifuge tubes (Costar Snap Cap; Corning Costar Europe, Badhoevedorp, The Netherlands) with a battery driven pellet pestle (Kontes, New Jersey, USA) and freeze-thawed three times before being inoculated on Vero cell monolayers and incubated for five days as described above. Cultures were then freeze-thawed three times, cell debris was pelleted, and 100 μl of the supernatant was added to 5 ml culture medium for the second passage. This was repeated once, to a total of three passages. A PCR with primers within the thymidine kinase gene (TK) and southern blotting and hybridization with radioactive probes from the TK-gene with a sensitivity of detecting 10 fg of vaccinia virus, corresponding to about 40 viral particles, were subsequently

TABLE 2. Species distribution of small rodents and common shrews (*Sorex araneus*) with antiorthopoxvirus antibodies.

Species	Competition-ELISA: % inhibition		IF[a]	PCR[b]
	>80%	>90%		
Bank vole[c]	15/69 (22)[d]	12/69 (17)	12/69 (17)	9/69 (13)
North. red-backed vole	1/9 (11)	0/9 (0)	0/9 (0)	3/9 (33)
Grey-sided vole	2/13 (15)	0/13 (0)	0/13 (0)	3/13 (23)
Wood mouse	42/81 (52)	23/81 (28)	13/81 (16)	9/81 (11)
Root vole	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)
Field vole	1/3 (33)	1/3 (33)	1/3 (33)	1/3 (33)
Common rat	2/2 (100)	2/2 (100)	0/2 (0)	NT
Common shrew	5/7 (71)	3/7 (43)	—	2/7 (29)
Norway lemming	24/34 (71)	19/34 (56)	—	0/14 (0)

[a] Immunfluorescence. A titer >10 was considered positive. Results not comparable (—).
[b] Polymerase chain reaction results from the same animals (T. Sandvik, unpublished data); NT, not tested.
[c] See text for scientific names of mammal species.
[d] Number of seropositive individuals/number of tested individuals (percentage of seropositive individuals).

used on DNA-extracts from the cell cultures in order to detect orthopoxvirus DNA.

Histological examination and electron microscopy

Tissue samples from lung, liver, spleen and kidney of the same 11 PCR-positive animals as above were examined for eosinophilic intracytoplasmic inclusion bodies in haematoxylin-eosin stained sections with a Leitz Laborlux S microscope (Wild Leitz GmbH, Wetzlar, Germany). Samples also were fixed in McDowell's fixative containing 4% (w/v) paraformaldehyde and 1% (w/v) glutaraldehyde, pH 7.2, embedded in plastic (Epon/Araldite; Boehringer Ingelham Bioproducts Partnership, Heidelberg, Germany), prepared for transmission electron microscopy, and examined in a JEOL-1010 transmission electron microscope (JEOL Scandinaviska, Stockholm, Sweden) operated at 80 kv.

RESULTS

Competition ELISA

Results from the ELISA screening of rodents and shrews are summarized in Table 1 (regional distribution) and Table 2 (distribution within species). Inhibition of >80 and >90%, relative to the rabbit hyperimmune serum, is compared with IF and PCR results. Of the sera examined, three bank voles, two wood mice, one field vole and one common rat had inhibition levels above the hyperimmune serum from the immunized cat (112%), the highest being 147% in a wood mouse. With support

from the IF results, sera which gave an inhibition of >90% were considered positive for anti-orthopoxvirus antibodies. Anti-orthopoxvirus antibodies were found in bank voles, wood mice, field voles, common rats, Norway lemmings, and common shrews. No differences were indicated with regard to sex. For bank voles, individuals with inhibition levels >90% were detected in 1993, 1994, and 1995; almost all of them were caught in May or June. Ten of 11 bank voles with known body weights weighed >18.0 g, and were considered to be overwintered adults at that time (Wiger, 1979). All the common field voles were caught in the autumn of 1995, and about 50% had body weights <18.0 g, which indicate that they were born during the spring or summer the same year. All the Norway lemmings were caught in autumn 1994, during a peak in the population. In Valdres (Fig. 1, location 8), 18 of 20 individuals had inhibition levels >90%. The weights ranged from 16 to 78 g, but the majority were subadult individuals born the same year. At the other trapping location for lemmings (Fig. 1, location 5) only 1 of 14 animals, with weights ranging from 20 to 70 g, had serum titres which reached this level of inhibition.

The preparations of the IgG fractions from the different rodent species and the

rabbit, were tested in the competition ELISA. They gave inhibition levels of 62%, 65%, 68%, and 136% for wood mouse, bank vole, Norway lemming, and rabbit, respectively, when compared to the original rabbit hyperimmune serum.

Immunofluorescence

The prevalence of anti-orthopoxvirus antibodies for the 12 of 69 (17%) bank voles were the same as the prevalence found by competition ELISA (Table 1, 2). Eight of 12 individuals seropositive in the ELISA (inhibition >90%), were seropositive by IF (titers 10 to >80). Three additional individuals seropositive in the IF assay, had inhibition levels from 83 to 87%, and were thus considered seronegative in the ELISA. This demonstrates that the level of anti-orthopoxvirus antibodies detectable in the IF-test, are comparable with the 80 to 90% inhibition level in the competition ELISA.

For the wood mice, 13 of 81 individuals had titers varying from 10 to 40, but only two of these animals had inhibition levels >90% in the ELISA. One of three field voles had an IF titer >40 and had an inhibition level in the ELISA of 115%. All the Norway lemmings, the common rats, and the shrews were negative in the IF-assay. Specific fluorescence was not detected for the rabbit preimmune serum, whereas the hyperimmune sera from rabbit and domestic cat, immunized with inactivated vaccinia virus and infectious cowpox virus, respectively (positive controls), both had IF titers of 320.

Virus isolation and PCR on cell cultures

No CPE was observed 5 to 7 days after inoculation of tissue samples on Vero cell monolayers from either of the three passages performed. Polymerase chain reaction and southern blotting and hybridization with radioactive probes failed to detect orthopoxvirus specific DNA in DNA extracts from the cell cultures.

Histological examination and electron microscopy

No eosinophilic intracytoplasmic inclusion bodies characteristic of cowpox virus infection were demonstrated in any of the samples examined. By transmission electron microscopy, two separate tissue samples from the four different organs from each of the 11 PCR-positive individuals were examined. No orthopoxvirus particles were detected.

DISCUSSION

In this study, serum samples from eight rodent species and from the common shrew in Norway were examined for anti-orthopoxvirus antibodies. Different types of ecosystems were represented, varying from deep inland forest to coast, from mountain regions with a small species diversity to warmer low-latitude regions, and from northern, southern, eastern and western parts of Norway. The animals were caught during a few days at each location and in very restricted areas, and for the local population of bank vole in Kongsvinger (69 sampled), wood mouse in Austrheim and Kalandsvatnet (18 and 55 sampled, respectively) and the Norway lemmings at Hardangervidda and Valdres (14 and 20 sampled, respectively), the numbers of individuals sampled are considered large enough to be representative to the respective local populations when estimating the seroprevalences.

A competition ELISA which eliminated the need for anti-species antibodies was used for rapid screening of 221 sera from the different species. Due to immunological crossreactivity between the orthopoxvirus species (Fenner et al., 1989b), this assay can not differenciate between antibodies directed against the different orthopoxviruses, but cowpox virus is considered as the most likely candidate because of two reported clinical cases and detection of orthopoxvirus specific DNA in rodents and shrews. Ectromelia virus, an important "pest" virus in laboratory mice, is another possible candidate as cause of the anti-orthopoxvirus antibodies. Ectromelia virus

has been isolated from wild rodents, but these animals may have been in contact with laboratory mice, and a wildlife reservoir is not evident (Fenner, 1994). Its occurrence as a pathogen of red foxes and mink (*Mustela lutreola*) in the Czech Republic has shown that hosts other than rodents are susceptible (Mahnel et al., 1993). However, a recent study indicated that bank voles are relatively resistent to both ectromelia virus and vaccinia virus, and confirmed that bank voles and wood mice are susceptible to cowpox virus (Bennett et al., 1997).

All the sera were also tested by an IF assay. For the bank vole sera, there was little discrepancy between the 90% inhibition level in the ELISA and the results from the IF-assay. With few exceptions, the bank voles with inhibition levels above 90% in the ELISA had IF titres varying from 10 to >80. With this information, we estimated an inhibition level of 90% compared to the rabbit hyperimmune serum to be the criterion for a test serum to be regarded as seropositive, i.e. to contain anti-orthopoxvirus antibodies. This high level of inhibition as the criterion for specific reactions lends a high degree of specificity to the test. However, this may decrease the sensitivity, and the demonstrated prevalence of anti-orthopoxvirus antibodies in bank voles (17%), woodmice (28%) and Norway lemmings (56%) should be considered minimum figures.

Testing the IgG preparations from wood mouse, bank vole, Norway lemming and rabbit sera in the ELISA gave restricted inhibition levels for the rodents, whereas an increase in inhibition level could be registrated for the rabbit. Due to different starting volumes of the different sera, and a high variability in the affinity of IgG for the different species and for different subclasses of IgG to protein A (Lindmark et al., 1983), in addition to several dilution steps during the preparation, these inhibition levels are not comparable. However, in spite of restricted affinity of IgG from wood mouse, bank vole and Norway lem-

ming to protein A, considerable inhibition was demonstrated, indicating that the ability of these sera to perform in the ELISA is a specific character of IgG.

The prevalences found for bank voles, wood mice and Norway lemmings are higher than the 8 to 12% seropositivity considered to be necessary for a particular species to function as a reservoir for poxviruses (Baxby, 1977; Crouch et al., 1995), although additional considerations must be taken along with the prevalence. The role of bank voles as a reservoir for orthopoxviruses is further indicated by the fact that nine of 12 individuals with inhibition levels >90% were adults caught in May, suggesting that they may have been infected through the winter and that newborns are infected by the adults during or after birth. Another possibility is that the infection is congenitally transmitted as reported for ectromelia virus in foxes and mink in the Czech Republic, where the adults seems to have a subclinical or mild disease, while the virus causes reproductive disorders with a mortality of 60% (Mahnel et al., 1993). Congenital transmission has also been reported for swinepox virus in Europe (Borst et al., 1990; Paton et al., 1990).

Individuals with inhibition levels >90% also were detected among wood mice, common rat, and common shrew, but not among root, northern red-backed or greysided voles. However, very few individuals were tested from these species, and no definite conclusions can be drawn with regard to their role as virus reservoirs in nature or the presence of orthopoxvirus in the species for which anti-orthopoxvirus antibodies were not detected. In fact, orthopoxvirus DNA has been detected in individuals of northern red-backed and greysided voles, demonstrating that orthopoxviruses do circulate in these two species as well (T. Sandvik, unpublished data).

By comparison of the results from the competition ELISA and the IF assay, it becomes clear that only the bank vole data are concurrent. Considerable discrepancies exist concerning the sera from wood

mice, and no seropositive individuals were detected among Norway lemmings and shrews by IF. The most probable explanation for this is that the commercial anti-species antibody conjugates detect antibodies of the wild rodents and shrews to varying degrees. The anti-mouse conjugate as second antibody has been shown to work very well for bank vole sera. However it gives high background reaction when testing sera from wood mice in a direct ELISA. A mixture of antimouse and antirat antibody conjugates is able to detect wood mice antibodies but the staining is often weaker than for bank voles.

There are several discrepancies between the serological results and the PCR results. In northern Norway (Fig. 1, location 1) no antibodies could be detected in northern red-backed and grey-sided voles (ELISA and IF), in spite of the fact that orthopoxvirus-specific DNA was detected by PCR in three of nine and two of nine individuals, respectively. These individuals may have been recently infected, not yet producing antibodies at the moment of capture and sampling. Anti-orthopoxvirus antibodies were detected in populations of bank vole and wood mouse in Søgne in southern Norway (Fig. 1, location 6), in which orthopoxvirus DNA could not be detected. A possible explanation for this is that such individuals may be convalescent individuals which have cleared the virus from their bodies. Alternatively, virus may be present in other tissues than those tested by PCR.

The fact that orthopoxviruses have never been isolated from seropositive wild rodents in western Europe in spite of several attempts, and that we were not able to isolate virus from orthopoxvirus DNA containing organs, may seem a bit confusing. Due to the sensitivity of the PCR and southern blotting and hybridization techniques used on DNA extracts from cell cultures inoculated with samples from PCR positive individuals, it is likely that no replication of orthopoxvirus had taken place. A possible explanation for this is

that orthopoxvirus particles present already was neutralized by antibodies. Furthermore, the PCR detects DNA and this in itself does not necessarily mean that infectious virus particles are present. Although the PCR products were of expected size (339 basepairs; bp) compared to vaccinia virus (strain Western Reserve) and the reference strain for cowpox virus (strain Brighton, ATCC number VR-302) and were verified by southern blot and hybridization with a 226 bp radioactive probe (Guanin + Cytosin = 34%) under stringent conditions, there is a possibility that the PCR products were non-specific, as they were not subjected to sequencing or endonuclease digestion. Although not likely, this might be an explanation for the lack of virus isolates from PCR positive samples.

As demonstrated by this and other studies, orthopoxviruses are widely distributed in rodent and shrew populations in Norway. Little is known about the role of these viruses as pathogens in their hosts species, whether latent or persistent infections are established, and how the viruses are being transmitted. Rodents have been shown experimentally to be susceptible to orthopoxvirus infections. They are widely distributed and have overlapping ecological niches with other animal species including man. They would hence be very efficient reservoirs for orthopoxviruses like cowpox virus. It is easily conceivable that wild and domestic carnivores might become infected from time to time by hunting and eating these small mammals. In this connection it is noteworthy that in the areas where a human and a feline case of cowpox virus infection appeared, the prevalence of anti-orthopoxvirus antibodies in wood mice were 28% and 18% (Fig. 1; locations 3 and 4, respectively).

Due to the distribution of orthopoxvirus antibodies in wild mammals reported here, it seems likely that cowpox virus infections in animals are under-reported. It also is remarkable that only one human case is reported. The distribution of orthopoxvi-

ruses in the Norwegian fauna should have an impact on considerations concerning the use of recombinant poxvirus-vectored vaccines for man, domestic animals and wildlife in Norway, since use of such vaccines must be regarded as a release of infectious virus. Recombinant poxviruses may represent hazards to immunosuppressed individuals, and the possibility of spontaneous recombinations with naturally occurring orthopoxviruses resulting in progeny with altered characteristics can not be excluded at this stage.

ACKNOWLEDGMENTS

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