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OCCURRENCE OF ATYPICAL MYXOMATOSIS IN CENTRAL EUROPE: CLINICAL AND VIROLOGICAL EXAMINATIONS

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An outbreak of the atypical form of myxomatosis struck a rabbit farm in Hungary. The animals had previously been vaccinated with a vaccine containing Shope rabbit fibroma virus strain. The disease appeared in winter when the presence of mosquitoes and fleas is not common. The virus was isolated from an eyelid specimen of a naturally infected rabbit. The surviving animals were observed for four weeks, blood samples were collected and, after euthanasia, organ specimens were also examined by morphological methods including pathology and electron microscopy. Serum samples were examined by virus neutralisation for antibodies. Genetic analysis of the isolated virus was carried out by polymerase chain reaction (PCR) and direct sequencing. The primers were designed on the basis of the major envelope gene (Env) of the Lausanne reference strain in the GenBank. The viral proteins were examined by SDS-PAGE. The isolated virus (ref. no.: BP04/2001) was able to infect the susceptible animals directly, by contact. The disease was characterised by respiratory symptoms of the upper tracheal tract, conjunctivitis and high mortality by the 11th–14th day. Aerogenic infection with strain BP04/2001 resulted in 100% morbidity among the susceptible animals. Sequencing of the amplified 400-bp-long DNA revealed 97% homology with the Env gene of the Lausanne strain, which proves that strain BP04/2001 is a variant of the Lausanne strain having been enzootic throughout Europe. The live vaccine strain used in Hungary against myxomatosis, which is also a Lausanne-derived strain, protected the animals. According to the protein analysis a protein of 200 kDa in size is not expressed in strain BP04/2001. This is the first report on atypical myxomatosis in Central Europe. The virus spreads by airborne transmission and may cause severe losses in the rabbit population.

Key words: Myxoma virus, myxomatosis, atypical, PCR, spread

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Myxoma virus (MV), a member of the family *Poxviridae*, is the causative agent of myxomatosis, a fatal disease of the European *Oryctolagus cuniculus* population. Myxoma virus has a large double-stranded DNA genome of 163 kilobases (kb), which replicates in the cytoplasm of infected cells (Moss, 1996). The virus has two main geographic types: South American (natural host: *Sylvilagus brasiliensis*) and Californian (natural host: *Sylvilagus brachmani*). In the natural hosts the native virus causes a benign cutaneous fibroma (Kerr and Best, 1998), but both types of myxoma virus are lethal to European rabbits.

The disease was first reported in Uruguay in 1898 and identified as a poxvirus in 1927 (Fenner, 1994). The South American viruses were deliberately introduced in the early years of the 1950s into Australia, then in 1952 into France, and they soon became enzootic in Europe (Manninger, 1956; Arthur and Louzis, 1988). The disease was first reported in Hungary in 1959 (Vetési, 1990).

Two forms of the disease have been identified: the myxomatous and the amyxomatous form. The previous one is further classified into classical (Széky and Benedek, 1969) and nodular (Benedek, 1968) forms. The amyxomatous form is sometimes referred to as atypical or chronic respiratory form (Marlier et al., 2000). The most characteristic features of the classical myxomatous form are the mucoid cutaneous tumours with swelling of the head and face, which is termed 'myxomatosis' referring to the mucoid nature of the cut surface of lesions (Benedek, 1968). A further feature is the severe impairment of the immune system resulting in uncontrolled secondary Gram-negative bacterial infections (Marlier et al., 1999). The myxomatous form is transmitted by blood-sucking arthropod vectors such as mosquitoes and fleas (Benedek, 1968). Transmission is passive, the virus does not replicate in the vector, only adheres to its mouth part (Fenner and Ratcliffe, 1965). The mortality rate is between 20 and 100%, depending on the virus strain (Marlier et al., 1999).

In contrast to the myxomatous form, atypical myxomatosis is not associated with cutaneous lesions. More or less intense respiratory syndromes are the major clinical signs (Table 1). It might be thought that atypical myxomatosis would not spread via vectors (Boucher and Nouaille, 2002). This kind of myxomatosis has been reported in France (Brun et al., 1981; Joubert et al., 1982) and in Belgium (Marlier and Vindevogel, 1996) to date. The origin of the atypical strains is unclear. Regarding the origin three hypotheses are present: *(i)* introduction of Californian strains into Europe, because the symptoms caused by Californian strains in European rabbits are very similar to those caused by the atypical strains; *(ii)* viral mutation of the South American strains having been present in Europe for 50 years (Arthur and Louzis, 1988); *(iii)* a possible link between the SG33 vaccine strain and the occurrence of atypical myxomatosis (Brun et al., 1981).

Table 1

Comparison of the main features of the classical and the amyxomatous forms of myxomatosis

	Atypical myxomatosis	Classical
Transmission	aerosol	vector
Incubation (day)	6–14	4–10
Clinical signs	conjunctivitis, dyspnoea	'myxomas'
CPE on RK-13 (day)	4–5	5–6
Mortality	83%*	20–100%**
Nucleic acid similarity in env gene	0.97	1.0 (Lausanne ref. strain)

*according to our animal experiments, **Marlier et al., 1999

In this study the first detailed observation on an atypical MV strain in Central Europe is reported. The disease appeared in a vaccinated rabbit population in 2000–2001. It is interesting that it occurred in wintertime, when mosquitoes are not common. The vaccine used in that rabbit flock contained a Shope rabbit fibroma virus strain.

Studies were carried out in order to *(i)* reveal the nature and the progression of the disease; *(ii)* isolate the virus and *(iii)* study the agent genetically.

Materials and methods

Organ specimens

Tissue samples of upper eyelid, lung, rectum and the mammary gland originating from a rabbit dying of natural infection in April 2001 were analysed. One-g amounts of each tissue sample were homogenised in 8 ml phosphate-buffered saline (PBS). The homogenate was mixed with penicillin, streptomycin and gentamicin (30 IU/ml each).

Isolation and titration of the virus

In order to isolate the virus, 2 ml of organ homogenates were inoculated on RK-13 cell monolayer in 50-ml flasks (Greiner Ag, Kreismünster, Austria). After absorption for 30 min, the homogenate was removed by washing twice with medium MEM Earle (Sigma-Aldrich, MO, USA), and the cells were incubated at 37 °C and observed daily. On day 5 after inoculation syncytia were observed on RK-13 cells. The samples showing no cytopathic effect (CPE) were passaged three times. The isolate from the eyelid was designated BP04/2001 and stored at –80 °C. The third passage of strain BP04/2001 was used for experimental infection. Titration was carried out by determination of the tissue culture infective dose on RK-13 cells. The titre of the virus was $10^{4.5}$ $TCID_{50}$/0.1 ml.

Animals

Fourteen New Zealand White rabbits were used for the animal experiments. They were 12–16 weeks old and weighed 1.5–2.2 kg. The animals were kept in isolators (Montair Andersen, Netherlands), with free access to feed and drinking water. Average room temperature was 20 °C. Prior to the trial a permit was obtained from the Animal Welfare Committee of the Institute for Veterinary Medicinal Products in accordance with the Animal Welfare Act XXVIII (1998).

Animal experiments

Two animal experiments were carried out. In order to obtain data regarding spreading and symptoms, three non-vaccinated rabbits were exposed to 40 cm^3 aerosol with strain BP04/2001 and three days later one contact control animal was housed together with them in Experiment A.

In Experiment B eight rabbits were vaccinated against myxomatosis with Myxovac (Ceva-Phylaxia, Budapest). This vaccine consists of an attenuated, Lausanne-derived strain. Two weeks later two groups were formed with four animals in each. Group I was infected with an amount of 0.15 ml virus fluid intradermopalpebrally (idp) and Group II with aerosol of BP04/2001, respectively. Two unvaccinated animals were used as controls (Table 2). Challenge was carried out in the isolator in order to maintain the special circumstances, which did not allow the presence of the usual vectors of MV, like mosquitoes and fleas. Marshall (1959) showed that ambient temperature could modify the clinical manifestation of the disease. In order to avoid this effect, the isolator was kept at 20 °C during the experiments.

Table 2

Animal experiment B. Eight rabbits were vaccinated with Myxovac, and then four animals were challenged with BP04/2001 using 40 cm^3 aerosol or 0.15 ml amount intradermopalpebrally

Rabbit groups	Infection		Protective effect
Vaccination IM	IDP	4 animals	3/4
	Aerogenic	4 animals	4/4
Control	IDP	1 animal	0/1
	Aerogenic	1 animal	0/1

The rabbits were observed daily for clinical signs throughout a 6-week observation period. Animals that died during the experiments were subjected to gross pathological examination. Organ samples (rectum, mammary gland, palpebra, spleen) were taken for virus isolation, electron microscopy and PCR. After 6 weeks all animals were euthanised.

Isolation of DNA

Tissue homogenates of eyelids were used for PCR. The samples were centrifuged at 5000 × *g* for 5 min and 200 µl of supernatant was subjected to Trizol™ DNA extraction. The precipitated DNA was pelleted at 12,500 × *g* for 15 min, washed once with 400 µl of 70% ethanol, dried and dissolved in 25 µl ddH_2O.

PCR and sequence analysis

A short fragment (400 bp) of the major envelope gene was amplified. The primers (For: GCC AAA CGA TAC ATC CAC; Rev: GGG GGA ATC TGA TAA AAA C) were designed on the basis of the Lausanne reference strain available in the GenBank (accession number: NC001132). The reaction was carried out in 50 µl volume which comprised 5 µl 10 × PCR buffer (100 mM Tris-HCl, pH 9.0, 500 mM KCl and 1 mg/ml BSA), 3 µl $MgCl_2$ (25 m), 0.5 µl of each dNTP (each 10 mM, Pharmacia), 20 pmol of each primer, 2 U Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA), 5 µl DNA, ddH_2O up to 50 µl and 1 drop of mineral oil (Sigma). Amplification was carried out in aGeneAmp PCR System 2400 (Perkin Elmer Cetus, Norwalk, CT, USA) using 35 cycles of 94 °C for 15 sec, 55 °C for 30 sec, 72 °C for 1 min and finally 72 °C for 7 min.

For visualisation, 8 µl of the PCR products were electrophoresed in 2% agarose gels. After electrophoresis, the gels were stained in ethidium bromide and viewed under ultraviolet light. The PCR products were sequenced by Genotype GmbH (Germany), the nucleotide and the deduced amino acid sequences were aligned with the aid of BioEdit 5.0.9 program (North Carolina State University), using the Clustal method (Verhofstede et al., 1996).

Accession number of sequence data

The nucleotide sequence data reported in this paper have been submitted to the GenBank sequence database and have been assigned the accession number AY136665.

SDS-PAGE

The isolated virus and the vaccine virus as control were propagated on RK-13 cells. The supernatant was centrifuged at 30,000 *g* for 12 h. The pellet was dissolved in 1 ml PBS, and treated with lysis buffer. The proteins were electrophoresed at 200 V for 2 h using 10% sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gel. Both high and low molecular weight markers were used (Sigma-Aldrich, MO, USA). After electrophoresis, the gel was stained with 0.125% Brilliant Blue R-250 (Sigma-Aldrich, MO, USA) for 1 h, then washed overnight.

Electron microscopy

One-mm^3 organ specimens from the eyelid, lungs, rectum and mammary gland of a carcass from a natural case were fixed for 3 h at 4 °C with a solution of 4% paraformaldehyde in 0.2 M phosphate buffer, pH 7.3, and of 0.2% glutaraldehyde, pH 7.3. The cells were washed four times in 0.2 M PBS, then post-fixed with 1% OsO_4 in phosphate-buffered saline for 2 h, rinsed in 0.2 M PBS, dehydrated in an ethanol series, and embedded in Durcupan resin (Electron Microscopy Sciences, Fort Washington, PA). The Durcupan polymerisation took 48 h at 56°C. Ultrathin (40–60 nm) sections were cut with an ultramicrotome (Reichert OM U3) and mounted on uncoated copper grids. Thin sections were imaged at 80 keV with an electron microscope (JEM JEOL 100S, JEOL Ltd., Akashima, Japan).

Results

The virus originated from a case of natural infection and it was isolated from the tissues of eyelid, lungs and rectum of an infected rabbit. The isolate designated BP04/2001 replicated well on RK-13 cell monolayers with a massive cytopathic effect, such as the formation of syncytia. Electron microscopy revealed poxvirus-like brick-shaped particles, 250 nm in diameter, in the cytoplasm (Fig. 1). The virus isolated from the eyelid was used for challenge.

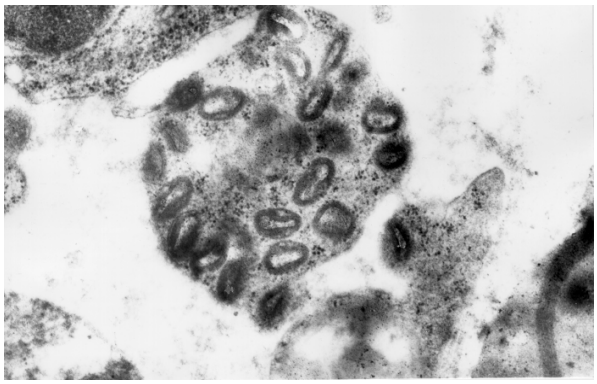

Fig. 1. Ultrathin section of an eyelid specimen by electron microscopy: Poxvirus-like virions in the cytoplasm

After 6–14 days all four animals in Experiment A showed symptoms of severe blepharoconjunctivitis and respiratory distress (Fig. 2). Florid skin lesions characteristic of myxomatosis were missing; however, necrotic spots on the surface of the skin did occur. Oedematous swelling of the eyelids, scrotum and anorectal area was observed.

Fig. 2. Severe conjunctivitis in a rabbit infected with BP04/2001

Spreading of the virus took place within the incubator, where no vectors, mosquitoes or fleas were present. The contact control and two rabbits infected with aerosol died.

In Experiment B, immunisation with the myxomatosis vaccine used in Hungary gave complete protection against aerogenic infection and 75% protection against intradermopalpebral infection, respectively. Both unvaccinated controls died of myxomatosis.

Sequences of the amplified DNA showed 97% similarity to the Lausanne strain, a characteristic member of the South American myxomatosis virus group. As this small segment of the huge MV genome did not allow a deeper insight into the genome of the virus, the SDS-PAGE protein profile was examined. The protein profile of the virus revealed that a protein of approx. 200 kDa in size was missing from strain BP04/2001 compared to the electrophoretogram of classical strains.

Discussion

Myxomatosis has been present in Europe for at least 50 years, causing severe economic losses to rabbit producers. Over that period of time the profile of the disease has changed. Production of proliferative skin lesions, 'myxomas' and nodules were the hallmarks of the European strains (Fenner and Ratcliffe, 1965). The appearance of the atypical form of myxomatosis may be a step in the evolution of MV. Atypical cases have been reported in France and Belgium to date (Brun et al., 1981; Marlier and Vindevogel, 1996). In the present study, the first appearance of atypical myxomatosis in Central Europe is reported. The clinical

signs caused by the virus isolated in the outbreak resemble those reported by Joubert et al. (1982) and Arthur and Louzis (1988) as atypical form of myxomatosis. The disease manifested in conjunctivitis, respiratory distress and orchitis. No 'myxomas' were observed. Although the number of animals involved in experiments was low, mortality from the atypical form may be higher (83%) than that caused by the classical form. Myxomatosis usually spreads via arthropod vectors, mosquitoes and fleas (Fenner and Ratcliffe, 1965); however, other forms of transmission could occur in intensive closed rabbitries, especially during the cold period of the year when there are few insects. Marlier et al. (1999) reported that atypical MV strains were recovered from some naso-conjunctival samples. In the sub-antarctic region Chapuis et al. (1994) reported transmission of myxomatosis without vectors. In the present study, we observed that the virus was able to spread directly from rabbit to rabbit. In animal experiment A, infection was carried out by aerosol in isolator, in order to exclude transmission by arthropods. The animals including the contact, uninfected control developed atypical myxomatosis indicating direct transmission of the virus among the animals.

The atypical form appeared in a vaccinated rabbit population, where a vaccine containing a Shope rabbit fibroma strain was used. In animal experiment B, the efficacy of the homologous vaccine was tested, which contains a MV strain and has been used in Hungary against myxomatosis. This vaccine provided efficient protection against the atypical form.

The origin of atypical myxomatosis is still unclear. Sequence analysis of the amplified region 400 bp in size revealed 97% similarity between the BP04/2001 and the Lausanne strain. This is interesting considering the fact that the Lausanne strain is the most characteristic member of the South American type, which is enzootic in Europe.

Many efforts were made to find genetic differences between the classical MV and the atypical MV, which could explain the different clinical signs. However, the molecular basis for the different clinical manifestations has not been clarified so far. To obtain indirect information about the genome of isolate BP04/2001, the protein profile was examined by SDS-PAGE. The protein profile of the isolated virus revealed that a protein approx. 200 kDa in size was missing compared to the classical MV. The expression of this protein could be impaired either by gene deletion or point mutation of gene resulting in internal stop codon. This question needs further examinations.

In summary, we reported here on an atypical myxomatosis case first in Central Europe. It has been observed that MV was able to spread via air in special isolated experimental circumstances, which excluded the presence of putative vectors of myxomatosis. Considering the high virulence and airborne spread, in the rabbit population of rabbitries attention must be paid to respiratory distress occurring during the cold period of the year when classical myxomatosis is not characteristic.

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