RECURRENT OUTBREAKS OF AMYXOMATOUS MYXOMATOSIS IN A COMMERCIAL RABBITRY ATTRIBUTED TO CONTACT TRANSMISSION

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ABSTRACT

An outbreak of atypical form of myxomatosis has struck a rabbit farm in Hungary. The disease appeared in winter when presence of mosquitoes and fleas is not common. The virus was isolated from eyelid specimen of a naturally infected rabbit. 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 way. The disease was characterised by respiratory clinical signs of the upper respiratory tract, conjunctivitis and high mortality by the 11-14 day after infection. Aerogenic infection with strain BP04/2001 resulted in 100% morbidity among the susceptible animals. Sequencing 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-derivated strain, protected the animals. According to the protein examinations a protein in size 200 kDa is not expressed in the strain BP04/2001. This is the first report on atypical myxomatosis in Central-Europe. The virus spreads in aerogen route and may cause severe losses in rabbit population.

Key words: myxoma virus, myxomatosis, amyxomatous, MV, PCR, spread.

INTRODUCTION

Myxomatosis is a specific disease of the European rabbit (*Oryctolagus cuniculus)* inducing severe losses in free-living wild and farmed rabbit populations as well. The causative agent is the Myxoma virus (MV), a member of the family *Poxviridae,* which includes several strains (Fenner, 1994) displaying characteristic tropism for skin or for oculo-respiratory tract, hence generating diseases with different clinical and pathological syndromes Two forms of the disease have been identified: the nodular (classical) form and the amyxomatous (respiratory) form.

The nodular (classical) form was recognised foremost, for which generalized mucoid cutaneous tumors proliferating above the skin surface are the major pathological clinical signs. The amyxomatous (respiratory) form has been reported almost three decades later on and only in France (BRUN *et al*., 1981; JOUBERT *et al.*, 1982), Spain (ROSELL *et al.*, 1984) and in Belgium (MARLIER and VINDEVOGEL, 1996) to date. In contrary to the nodular form, it is associated with highly reduced cutaneous lesions.
Regarding the spreading in case of amyxomatous myxomatosis, which does not present cutaneuos clinical signs, some different ways of transmission could be suspected. Supporting this idea BARCENA *et al.* (2000) found MV strains spreading among rabbits by contact transmission. In the sub-antarctic region CHAPUIS *et al.* (1994) reported transmission of myxomatosis without vectors.

In this study the first detailed observation on an amyxomatous MV strain in Central-Europe is reported. Furthermore studies were carried out in order to i) isolate the virus ii) observe the nature and the transfer of the disease within experimental conditions, iii) scrutinise the effectivity of vaccination and iiii) study the agent genetically.

MATERIAL AND METHODS

Field observations

The field observations were made in a large-scale intensive rabbitry (around 15 000 does) between 1999 and 2001. The hybrid rabbits were housed, caged and fed according to the general rules. Pests and insects were cleared regularly. Artificial insemination was performed for propagation.
At the periode when the myxomatosis virus was suspected to be brought into the farm more than 6 months has passed since the previous vaccination. From 1999 the myxomatosis vaccination was taken at 28 days of age and a booster after weaning (7 weeks of age) for the broilers, for breeding replacement animals at 18 weeks age and at every 4 months afterwards. The vaccine, application method and protocol used, however, have changed more times within the respective period.
Breeding animals were introduced into this farm from the grandparent unit in form of two days old sucklings adopted immediately by local does, or weaned rabbits after the requested quarantine period have been provided. In any other respect the farm was strictly separated from the outside.
Clinical signs and pathognomical lesions were observed during visits performed in the periods of outbreaks.

Organ specimens

Tissue samples of upper eyelid, lung, peri-annal tissues; with skin and the mammary gland with skin originated from a rabbit died in natural infection in February 2001 were analysed. The 1 g amount of each tissue sample were homogenised in 8 ml phosphate saline buffer (PBS). The homogenate was mixed with penicillin, streptomycin and gentamycin (30-30 IU/ml).

Isolation and titration of the virus

In order to isolate virus, 2 ml of homogenates of organ were inoculated on RK-13 cell monolayer in 50-ml volume flask (Greiner). After 30 minutes absorption, the homogenate was removed by washing twice with medium, and the cells were incubated at 37°C and observed daily. On day 5 after inoculation syncytia were observed on RK13 cells. The samples showing no cytopathic effect were passed three times. The isolate named BP04/2001 was stored at -80°C. Third passage of the BP04/2001 was used for experimental infection.

Animals

Fourteen commercial rabbits were used for the animal experiments in total. All were 8-10 weeks old, in weight of 1.5-2.2 kg. The animals were kept in isolator (Montair Andersen, Netherlands), unlimited access to food and water was ensured, the average temperature was 20°C. All care and experimental procedures were permitted by the Animal Welfare Committee of the Institute for Veterinary Medicinal Products.

Experimental infections

Two animal experiments were carried out:

In experiment A, three rabbits were exposed to 40 cm^3 aerosol of $10^{5.5}$PFU/ml BP04/2001 sprayed into the closed air-space of the isolator. One contact control animal were introduced to the isolator later, and housed together with one contact control animal in order to obtain data regarding the spreading and the clinical signs. Vectors were not present in the isolator neither on the rabbit's body. The temperature was held at 20°C during the experiments.

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 consisting of 4-4 animals. Group I was infected with amount 0.15 ml intradermopalpebrally (idp) and Group II with aerosol of BP04/2001, respectively. Two animals were used as unvaccinated controls.

The rabbits were monitored 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 histopathological examination, virus isolation and PCR. After 6 weeks all animals were euthanised.

Isolation of DNA

Organ specimens of eyelids were used for PCR. The samples were centrifuged at 5000 x g for 5 minutes and 200 µl of supernatant was subjected to Trizol™ DNA extraction. The precipitated DNA was pelleted at 12500 x g for 15 minutes, washed once with 400 µl of 70% ethanol, dried and dissolved in 25 µl ddH_2O.

PCR and sequence analysis

A short fragment (492 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 base of Lausanne strain available in the GenBank (accession number: NC001132). The reaction was carried out in 50 µl volume which comprised 5 µl 10x 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 a GeneAmp 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 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).

SDS PAGE

The isolated virus and vaccine virus as control were propagated on RK-13 cells. The supernatant was ultracentrifuged at 30 000 g for 12 hours. The pellet was dissolved in 1 ml PBS, which was treated with lysis buffer. The proteins were electrophorised on 200 V for 2 hours using 10% sodium dodecil sulfate polyacrylamide (SDS PAGE) gel. Both High and Low Molecular Markers were used (Sigma). After electrophoresis, the gel was stained with 0.125% Coomasie Blue R-250 (Sigma) for 1 hour, then washed overnight.

Electron microscopy

1 mm^3 organ specimens from the eyelid, lungs, rectum and mammary gland of carcass originated from natural case were fixed for 3 hours at 4°C with a solution of 4% paraformaldehyde in 0.2 M phosphate buffer, pH 7.3, and of 0.2% glutaraldehydrate, pH 7.3. The cells were washed 4 times in 0.2 M PBS, then postfixed with 1% OsO_4 in phosphate saline buffer for 2 hours, 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 hours at 56°C. Ultrathin (40-60 nm) sections were cut with an ultramicrotome (Reichet 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 AND DISCUSSION

Field observations on repeated outbreaks

The disease appeared first at December 1998 among susceptible animals following introduction of 2 days old sucklings from the grandparent breeding unit. First the adopting lactating females become infected and showed atypical clinical signs followed by mortality rate above 70%. Myxomatous nodules were entirely missing. Acute respiratory distress, serous nasal discharge and conjunctivitis were the features of the disease, manifestating most prominently among females, dying mainly from pneumonia. Based upon these uncharacteristic clinical signs and season of the outbreak the diagnostic procedures were concentrated on pasteurellosis. Indeed *Pasteurella multocida* was isolated from the pulmonary lesions, the antibioticum resistance was detected and antibiotic treatment implemented. For all this, suckling rabbits around 30-

32 days old and broilers started to became ill. For the sucklings and broilers the severe blepharoconjunctivitis closing completely their both eyes, and perianal swellings, which is not typical in this age group was found. Eventually the efforts to isolate MV leaded to the correct diagnosis, which was followed by emergency vaccination of the apparently healthy animals on the contaminated farm. The incidence of the disease had fallen immediately seen such as the farm were cleared by summer 1999. However, despite enormous efforts made to perfectly perform the newly established vaccination program recurrent outbreaks occured every year. These were some way uncharacteristic in term of slow progression and that most of the outbreaks occured at wintertime, when the mosquitos are not present. The breeding animals were not affected at all in these occasions and their average fertility rate was about 70%. The clinical signs detailed above appeared first among the sucklings at more and more earlier age, finally when they were only 23 days old, just emerging from the nestbox. Not all of these animals died soon, those displaying two-sided blepharoconjunctivitis have been exterminated. The mortality rate still remained under 5% in yearly average, only doubled in the outbreak groups. The remaining healthy looking animals were vaccinated according to the established schedule, but the diseases continued to appear in the respective group in the fattening period with a mortality rate about 16%, but below 6% in those, which were not involved. In every year the situation settled down to the summer months, when this disease was not diagnosed.

Virus isolation and transmission experiment

The virus originated from a case of natural infection and it was isolated from the tissues of eyelid, lungs and peri-anal tissues of an infected rabbit. The isolate named BP04/2001 was replicative on RK-13 cell monolayers with massive cytopathic effect, such as syncytia. Electron microscopy revealed poxvirus-like brick-shaped particles in the cytoplasm in diameter 250 nm (Fig 1). The virus isolated from eyelid was used for the animal experiment. The titration was carried out by the determination the tissue culture infective dose on RK-13 cell, the titer of the virus was$10^{5,5}$ PFU/ml.

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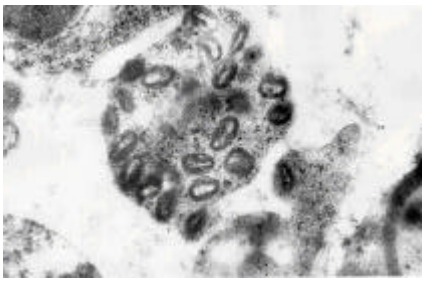

Figure 1.: Ultrathin section of an eyelid specimen by electron microscopy: Poxvirus-like virions in the cytoplasm.

After 6-14 experimental days all four animals showed symptoms of massive blepharoconjunctivitis, and respiratory distress (Fig 2). Florid skin lesions characteristic to myxomatosis missed, however, necrotic spots on the surface of the skin occured. Oedematous swelling of eyelid, scrotum and the anorectal area was observed.

The spreading of the virus took place in special isolated circumstances, which was free of the usual vectors of myxomatosis like mosquitoes or fleas. The contact control and two rabbits infected with aerosol died. According to the histopathological examinations the rhexis was the most characteristic lesion, and the inflammation has secondary importance.

Figure 2.: Severe conjunctivitis in the infected animals with BP04/2001.

Efficacy of Vaccination

The immunisation with the vaccine against myxomatosis used in Hungary has given complete protection for sensitive animals in case of aerogen infection and 75% of protection in case of idp infection, respectively. Both controls died in myxomatosis Table 1.).

Table 1: The 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 intrapalpebrally, respectively

GROUPS, N	INFECTION Method	N	PROTECTIVE EFFECT
Vaccination IM, 8	IDP	4	3/4
	AEROGEN	4	4/4
Control, 2	IDP	1	0/1
	AEROGEN	1	0/1

Genomic and protein characterisation

The genetic analysis of the sequence of the isolate revealed that on the examined region it has 97% similarity to Lausanne strain, the characteristic member of the South American group. However, this small segment of the huge MV genome has not allowed

deeper insight to the genetics of the virus, for this reason the purified and lysated virus was electrophoreted on SDS-PAGE gel. The protein profile of the virus revealed that a protein in size ca. 200 kDa is missing in case of BP04/2001 compared the electrophoretogram with that of a classical strains (Fig 3).

Discussion

In the present study, the first outbreak of the anodular myxomatosis in Central-Europe is reported. The disease emergence was first documented in an enclosed, large-scale commercial rabbitry where arthropodes were effectively controlled, moreover, the main outbreaks were observed at the winter season when the presence of arthropod vectors is again very unlikely. In animal experiment A, the infection was carried out by aerosol in isolator, in order to exclude the transmission by arthropods. The rabbits including the contact, uninfected control developed anodular myxomatosis indicating the direct transmission of the virus among the animals. Our experimental results unequivocally support the experiences in the commercial rabbitry providing the foremost scientific evidence of MV transmission in abscence of vectors.

On the involved farm outbreaks has been repeated for years, contrary to the implemented vaccination schedule. However asymptomatic periods were observed for as long as 9-10 months and during the outbreaks the mortality was lower and the general health status of the population regarding myxomatosis were on an acceptable level, although not excellent. This could not be explained by the vaccine inefficiency since the vaccine consisting of a MV strain and used in Hungary against myxomatosis gave efficient protection against this amyxomatous form, in animal experiment B.

Considering the experimental infection, clinical signs caused by the virus isolated in the outbreak highly resembled to those found on the respective farm and those of reported by JOUBERT *et al.* (1982) ARTHUR and LOUZIS (1988) and experimentally by MARLIER *et* al. (1999 and 2000) for amyxomatous form of myxomatosis. The disease manifested in conjuctivitis, respiratory distress and orchitis. No “myxomas” were observed. The mortality of the anodular form of myxomatosis in sensitive animals was much higher than that of the classical form and it is based on mainly the dyspnoe and conjunctivitis.

The origin of 5 amyxomatous MV strains was studied by MARLIER *et al*. (1999). Genomic restriction profiles undoubtedly demonstrated that those strains came from the Lausanne strain introduced into France in 1952 and not from a putative introduction of a Californian strain into Europe. The PCR assay carried out in our study to classify this isolate produced an amplified region in size 492 bp and 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 the South American type, which is enzootic in Europe.

Many efforts were carried out to find genetic differences between the classical MV and the amyxomatous MV, which could explain the different clinical pictures. However, the molecular basis for the different clinical manifestation has not been clear so far. To obtain indirect information about the genome of the anodular MV, the protein electropherogram was generated out by SDS-PAGE. Compared the protein profile of the anodular MV with the classical MV, a ca. 200 kDa protein is missing in case of the amyxomatous MV. The expression of this protein could be impaired either by gene

deletion or point mutation of gene resulting internal stop codon. This question needs further examinations.

CONCLUSIONS

The myxomatosis has been present in Hungary for at least 40 years, mainly appearing as the nodular type of the disease. During this time veterinarians and rabbit producers had learned to exert effective measures against the spread of MV in form of separation, quarantine, insect killing and vaccination. The appearance of the amyxomatous form of myxomatosis may be a step in the evolution of MV, but its unique features make essential the revision of main points of diagnosis and prevention. Based on our result the following conclusions could be postulated in summary:

1. In case of oculo-respiratory symptomes, especially blepharoconjunctivitis of both eyes, occurring during the cold period of the year when the classical myxomatosis is not characteristic MV detection is inevitable.
2. Good barrier technique and vector control is insufficient to protect susceptible animals from infection, since spread on aerogen route has been verified.
3. The result of the vaccination on contaminated farm could be inefficient in the view of eradication, however vaccination on free farms is presumably effective and inevitable for prevention.
4. Our results support the opinion of DUCLOS *et al.* (1986) who suggested first time that myxomatosis should be pursued in the investigation of respiratory infections in rabbit.

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