Ultrastructural Study of Poxvirus Causing Myxomatosis in Rabbits, in São Paulo and Santa Catarina, Brazil

Estudio Ultraestructural del Poxvirus Causal de Mixomatosis en Conejos,
en São Paulo y Santa Catarina, Brasil

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SUMMARY: The myxomatosis is a contagious worldwide disease caused by poxvirus which infects domestic and wild rabbits. In the present study we present two distinct outbreaks of myxomatosis when raising rabbits, one for commercial purpose of production of meat and skins and, another one for the commercialization of ornamental rabbits. The observed signs were ocular, auricular, nasal, testis lesions and many times scattered throughout the body of the animals. The lesions were characterized by formation of nodules that by palpation disclosed gummy or gelatinous aspect. At the transmission electron microscopy, all the skin and crust samples were analyzed by negative staining technique. A great number of particles with morphology similar to the poxvirus, some enveloped in a brick-shaped and irregular disposition of tubules on the external membrane, measuring 300x240 nm on the average were visualized. Ultrathin sections revealed the presence of intracytoplasmic inclusion bodies surrounded by membrane containing oval particles, measuring 270 x 130 nm, containing nucleus or an internal biconcave (dumbbell-shaped) core. Immature particles (empty), surrounded by membrane were also observed. In addition, intracytoplasmic electron dense inclusion bodies containing viral particles budding of dense amorphous material and intranuclear fibrillar or "digital" inclusions showing a regular striation and arranged in groups were found in the middle of granular material. The nuclei were deformed with densely condensed chromatin forming amorphous and electron dense inclusion bodies. In the immunocytochemistry technique, the antigen-antibody reaction was strongly marked by the particles of colloidal gold, emphasizing the viral particles. The techniques used in this study were important in the diagnosis of the affected animals.

KEY WORDS: Myxomatosis; Poxvirus; Rabbits; Transmission electron microscopy.

INTRODUCTION

Myxomatosis is a severely debilitating infectious-contagious disease that affects the wild and domestic rabbits (Fenner *et al.*, 1992). Etiological agent was first isolated from a colony of laboratory rabbits, in Uruguay, 1898 and characterized as a poxvirus only in 1927. In natural hosts of the leporid species, *Sylvilagus brasiliensis* in South America and *Sylvilagus bachmani* in California, the viral strains produce in adult animals benign fibroma and generalized disease in young animals. In the European rabbits, two forms of the disease have been identified, the nodular classic and the amyomatous or respiratory form (Fenner & Hatclife, 1965).

In nodular myxomatosis, after incubation period of 2-10 days, the first sign is the appearance of a nodule, in

general, in the ocular region which increases in size, becomes protuberant and ulcerates evolving to blepharoconjunctivitis and mucoid subcutaneous tumor formation in the face and ear, originating deformities that confer to the animal a leonine aspect. Additionally, small nodule tumors appear in extremities and genital organs (Fenner *et al.*) and orchiepididymitis causing infertility (Ferreira & Ferreira, 1990; Fenner & Fantini, 1994; Fountain *et al.*, 1997). The disease may evolve, between 48 hours and 12 days, to paralysis and death (Ferreira & Ferreira), being the estimated death rate ranging from 20 to 100% depending on the viral strain. In the recovery process, the lesions gradually heal (Arthur & Louzis, 1988). There are several vectors of transmission in the nodular form (Beer, 1999). In England,

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the rabbit's flea is the mayor transmitting agent, in some areas of Europe, the mosquitoes (Fenner & Fantini), in Mexico, the mosquitoes of the *Ceratopogonidae* and *Anthomyiidae* family (Luna, 2000), in Brazil, flies, fleas and mosquitoes *Aedes aegypti*, *Anopheles* and *Culex* (Ferreira & Ferreira). Airborne transmission is also reported in the literature, as well as by direct contact among enclosed rabbitries (Farsang *et al.*, 2003).

In the amyomatous form, the main symptom is in the respiratory tract which may evolve to pneumonia or bronchopneumonia besides the presence of small nodules in the skin (Fenner *et al.*; Marlier *et al.*, 2000). An outbreak of atypical myxomatosis characterized for respiratory symptoms of the superior tracheal tract associated to the conjunctivitis and high mortality was reported in the Hungry (Farsang *et al.*). The transmission of this form of disease occurs by direct contact among enclosed rabbitries. It is important to emphasize that this type of transmission was also detected in wild rabbits (OIE, 2008). As well, in mountain hares a contagious mucocutaneous dermatitis caused by a poxvirus was found (Saari *et al.*, 2005).

The myxomatosis was reported in various parts of the world, as Mexico, Australia, Spain, France and England (Shepherd *et al.*, 1978; Ross *et al.*, 1989; Ghram *et al.*, 1996; Gelfi *et al.*, 1999; Luna; Calvete *et al.*, 2002; Merchant *et al.*, 2003). In Brazil, myxomatosis occurs periodically and, in the last 20 years, only two outbreaks had been reported, both in Rio de Janeiro state, in rabbitries of small domestic producers, with high mortality (Barbosa *et al.*, 1996; Bruno *et al.*, 2004).

The poxvirus, member of the *Poxviridae* family, *Leporipoxvirus* genus is among the largest and most complex of all animal viruses. They do not possess nucleocapsid and the external membrane contains a central zone dumbbell-shaped and two lateral bodies of unknown nature. The irregular disposition of the tubules on the external lipoproteic bilayer confers it a textured characteristic appearance. Its replication occurs in the cytoplasm and the particles released by rupture of the cell acquired an envelope form (Doane & Anderson, 1987; Fenner *et al.*; Fenner, 1996, 2000; Diven, 2001).

The genome of myxoma virus, DNA of double strand of 162 Kbp (Cameron *et al.*, 1999) has a central region with structural and enzymatic genes highly conserved, essential to the maintenance of the functions of viruses (Upton *et al.*, 1990). The capsid is composed for 17 different proteins (Zachertowska *et al.*, 2006), being that the (MV) M-T5 gene codifies an important protein in the viral replication (Johnston

et al., 2005) and the M141 R gene a necessary protein to the development of a lethal infection in vivo (Cameron *et al.*, 2005).

The transmission electron microscopy has been widely used for many authors to detect typical particles of poxvirus (Tektoff *et al.*, 1971; Purcell & Clarke, 1972; Yuill, 1972; Marcato & Simoni, 1977; Barbosa *et al.*; Luna; Farsang *et al.*; Saari *et al.*; Ganière *et al.*, 2008), being one of the techniques more recommended by the OIE for the accomplishment of the laboratorial diagnosis of the virus, in suspension of skin lesion.

Considering effectiveness of this technique, our proposal was to detect the etiological agent in skin lesions of rabbits regarding the two outbreaks.

MATERIAL AND METHOD

Description of the outbreaks. The first outbreak was detected in 1978, in São Paulo, in the city of Itapeirica da Serra in a rabbitrie with 340 rabbits of Flandres Giant New Zealand White strain, whose production was for consume and the animal skin for industry. Among those, 26 adult animals presented the disease with conjunctivitis and rhinitis with a sanguineous mucous fluid and when dry it formed adherent crusts to nasal orifices (Fig. 1). The ocular status evolved to panophthalmitis with accentuated palpebral edema and by palpation, the periorbital region revealed a gelatinous texture. Some animals presented lesions on the paws and ear extremities with became edemaciated (Fig. 2). The testis of four male reproductive rabbits, were swollen and with a gelatinous texture (Fig. 3). Eleven out of the 26 sick rabbits went to death.

The second outbreak, in 2002, occurred in Santa Catarina, in the city of Sangão (Jaguariúna), in a rabbitrie with approximately 150 ornamental rabbits. Animals presented nystagmus, motor incoordination followed by anorexia, depression, opaque pelage, subcutaneous edema, conjunctivitis, otitis, rhinitis, face malformation, caquexy and death. Most of the young animals (0 to 60 days) went to death without showing signs of the disease. Some adult animals recovery but anorexia and cutaneous folds in the neck (chin) area remained. The mortality rate initially low, increased over the months, and only 20 animals remained but with contagious symptoms.

Electron microscopy techniques. Fragments of skin lesions and dry crusts were collected and sent to the Electron Microscopy Laboratory of the Biologic Institute, São Paulo,

Fig.1. Animal showing conjunctivitis and rhinitis and abundant mucosanguineous fluid and crust formations.

Fig.2. Animal with panophthalmia and rhinitis. Observe the ear markedly edematous with lesions along the border, edema in paws and lesion in the fingers

Brazil. They were processed for transmission electron microscopy using negative staining (rapid preparation), resin embedding and immunocytochemistry techniques.

Negative staining technique (rapid preparation). The negative staining process was performed according to the

methodology described by Brenner & Horne (1959), Hayat & Miller (1990) and Madeley (1997). In this technique the clinical samples were suspended in phosphate buffer 0.1 M and pH 7.0, placed in contact with metallic copper grids with carbon stabilized supporting film of 0.5% collodium in amyl acetate. Next, the grids were drained with filter paper and negatively stained at 2% ammonium molybdate, pH 5.0.

Resin embedding technique. Fragments of skin lesions and dry crusts were fixed in 2.5% glutaraldehyde in 0.1M, pH7.0 phosphate buffer and pos-fixed in 1% osmium tetroxide in the same buffer. After dehydration in cetic series, the fragments were embedded in Spurr resin (González-Santander, 1969; Luft, 1961). Ultrathin sections were cut on the LKB ultratome and mounted on copper grids. The sections were contrasted with uranyl acetate-lead citrate (Watson, 1958; Reynolds, 1963).

Immunocytochemistry technique. At the immunolabeling technique with colloidal gold particles for negative staining, the copper grids were placed in contact with viral suspension and, after removing excess with filter paper, the same were

Fig. 3. Testis impairment where we can observe increase in volume.

put on specific primary antibody drops. After successive washings in PBS drops, the grids were incubated in protein A drops in association with 10 nm gold particles (secondary antibody). Grids were then contrasted at 2% ammonium molybdate, pH 5.0 (Knutton, 1995). Observations were made in a Philips EM 208 electron microscope, at 80 kV.

RESULTS

Negative staining technique (rapid preparation). A great number of particles with morphology similar to the poxvirus,

some enveloped (Fig. 5, arrow) in a brick-shaped and irregular disposition of tubules on the external membrane (Fig. 4, arrow), measuring 280x230 nm on the average were visualized in all the samples of the fragments of skin lesions and dry crusts negatively stained at 2% ammonium molybdate.

Resin embedding technique. Ultrathin sections of the fragments of skin and crusts, positively stained by combination of uranyl acetate and lead citrate, revealed the presence of intracytoplasmic inclusion bodies (Fig. 6), surrounded by membrane and containing oval viral particles,

Fig. 4. Negatively stained poxvirus showing irregular disposition of tubules on the external membrane (arrow). Bar = 100 nm.

measuring 270 x 130 nm, with a nucleus or internal biconcave (dumbbell-shaped) core (Fig. 6, big arrow). Immature particles (empty), surrounded by membrane were also observed (Fig. 6, minor arrow). Intracytoplasmic electron dense inclusion bodies (Fig.7), containing viral particles (Fig. 7, lower arrow) budding of dense amorphous material (Fig.7, big arrow) were also observed. Intranuclear fibrillar or “digital” inclusions (Fig. 8, arrow), showing a regular striation and arranged in groups (Fig. 9, arrow) were found in the middle of granular material.

There were still a large number of intracytoplasmic vacuoles, surrounded by membranes, measuring between 480 and 1150 nm in diameter (Fig. 10). Some vacuoles contained granular material in its interior or on the external surface (Fig. 10, big arrow) and electron dense fibrils could be seen around some of the vacuoles (Fig. 10, minor arrow).

The nuclei were deformed with densely condensed chromatin forming amorphous and electron dense inclusions bodies (Fig.11, arrow).

Immunocytochemistry technique. In the immunocyto-chemistry technique, the antigen-antibody reaction was marked by the particles of colloidal gold, strongly enhanced the viral particles (Fig. 12, arrow).

Fig. 5. Negatively stained poxvirus showing enveloped particle (arrow). Bar = 130 nm.

Fig. 6. Ultrathin section of the fragments of skin. Intracytoplasmic inclusion bodies containing oval viral particles with a nucleus or internal biconcave (dumbbell-shaped) core (big arrow). Immature particles (empty), surrounded by membrane (minor arrow). Bar = 200 nm.

Fig. 7. Ultrathin section of the fragments of skin. Intracytoplasmic electron dense inclusion bodies, containing viral particles (lower arrow) budding of dense amorphous material (big arrow). Bar = 320 nm.

Fig. 8. Ultrathin section of the fragments of skin. Intranuclear fibrillar or "digital" inclusions (arrow). Bar = 600 nm.

DISCUSSION

In this study, rabbits with poxvirus presented signs and symptoms, such as conjunctivitis, subcutaneous and perineal edema, and facial malformation conferring the animal a leonine aspect, as reported in the literature (Ferreira & Ferreira; Corrêa & Corrêa, 1992; Fenner *et al.*; Barbosa *et al.*; Bruno *et al.*).

It was observed that the periorbital region as well as testis of the studied animals had a gelatinous texture.

The mucoid nature of the lesion surface caused by poxvirus confers the name of myxomatosis to the disease (Fenner & Ross, 1994).

Increase in volume of testis observed by us in some reproductive animals was also found by other authors (Ferreira & Ferreira; Fountain *et al.*; Bruno *et al.*).

Some animals of our study showed neurological signs as nystagmus and motor incoordination. Studies indicated that animals may present paralysis as well (Fenner *et al.*).

No typical respiratory symptoms of the myxomatosis form described by Farsang *et al.* could be observed as well as pulmonary lesions verified by Bruno *et al.*

The two outbreaks occurred in the summer time when there is a bigger proliferation of transmitting insects as fleas, flies and mosquitoes, as analyzed by Corrêa & Corrêa, Fenner *et al.* and Bruno *et al.* Mosquitoes as *Aedes aegypti*, *Anopheles* and *Culex* may act as virus reservoirs (Ferreira & Ferreira). Airborne transmission had already been reported by Farsang *et al.*

A great number of typical poxvirus particles, measuring approximately 300 x 240nm was detected by negative contrast technique for transmission electron microscopy (rapid preparation) in all the analyzed samples.

These morphological features are in accordance with other myxomatosis studies (Padgett *et al.*, 1964; Tektoff *et al.*; Strayer *et al.*, 1983; Barbosa *et al.*; Luna).

The use of 2% ammonium molybdate allowed the ideal electronic contrast to viral identification by the negative staining technique. This stain is also indicated by OIE.

By means of the resin embedding technique, we observed oval poxvirus particles, measuring 270 x 130 nm,

Fig. 9. Ultrathin section of the fragments of skin. Intranuclear fibrillar or “digital” inclusions showing a regular striation and arranged in groups (arrow). Bar: 170 nm.

Fig. 10. Ultrathin section of the fragments of skin. Intracytoplasmic vacuoles, surrounded by membranes contained granular material in its interior (big arrow). Electron dense fibrils around some of the vacuoles (minor arrow). Bar 1200 nm.

Fig. 11. Ultrathin section of the fragments of skin. Nuclei with densely condensed chromatin forming amorphous and electron dense inclusions bodies (arrow). Bar: 900 nm.

with a nucleus or biconcave (dumbbell-shaped) internal core. These aspects were also visualized by other authors (Tektoff *et al.*; Purcell & Clarke; Ogier *et al.*, 1974; Karstad *et al.*, 1977; Bohn, 1980; Brooks *et al.*, 1995; Saari *et al.*).

The presence of intracytoplasmic inclusion bodies, surrounded by a membrane, containing complete and incomplete viral particles is in accordance with other investigations (Tektoff *et al.*; Purcell & Clarke; Saari *et al.*). Additionally, we noted deformed nuclei with compacted dense chromatin forming electron dense and amorphous inclusions, intranuclear fibrillar or “digital” inclusions, showing periodical striation and disposed in groups among granular material as described in cases of swine variole and avianpox (Teppema & Boer, 1975; Bersano *et al.*, 2003; Catroxo *et al.*, 2005; Melo *et al.*, 2008).

In the immunocytochemistry technique, the intensive mark of poxvirus particles with colloidal gold allowed to confirm the poxvirus presence in the studied animals.

This technique was also used to mark particles of poxvirus in other cases of swine variole and avianpox (Bersano *et al.*; Catroxo *et al.*, 2005; 2006; Melo *et al.*, 2007; Catroxo *et al.*, 2008).

The electron microscopy which is extensively used is advocated as the elective method to detect virus in fragments of skin lesions (Fenner *et al.*; Fenner & Fantini; Robinson & Kerr, 2001; OIE).

The employed techniques were crucial to diagnose the diseases in the mentioned outbreaks.

It is evident that precarious conditions with lack of medical and sanitary procedures contribute to myxomatosis introduction and distribution in several regions (Ghram *et al.*).

Thus, to avoid the disease outbreak, control and prevention measures were established in rabbitries, where hygienization was recommended into the breeding sheds, applying nylon insect doors and windows screen all over the facility, construction of pedilavium to access the shed, being mandatory the use of rubber boots for all the people who work or have free entrance to rabbitries and restricting the entrance of foreign people.

The results of the present study were valuable in the control and prevention of outbreaks in rabbitries as well to be careful when observing any suspicious symptom on these animals.

Fig. 12. Immunocytochemistry technique. Poxvirus marked by the particles of colloidal gold (arrow). Bar = 120 nm.

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RESUMEN: La mixomatosis es una enfermedad contagiosa de distribución mundial, causada por poxvirus que infecta conejos domésticos y salvajes. En este estudio presentamos dos distintos surtos por mixomatosis que ocurrieron en producciones de conejos, una para fines comerciales de producción de carne y pieles y otra para el comercio de conejos domésticos. Las señales observadas fueron afecciones oculares, nasales, testiculares y, a veces, también distribuida por todo el cuerpo de los animales. Estas se caracterizaban por formación de nódulos que a la palpación tenían un aspecto gelatinoso o gomoso. En la microscopía electrónica de transmisión, por la técnica de contrastación negativa, se pudo observar en todas las muestras examinadas de piel y de costras, un gran número de partículas típicas de poxvirus, con envoltura y forma de ladrillo, mostrando disposición irregular de los túbulos sobre la membrana externa, midiendo 300 x 240 nm en el promedio. Cortes ultrafinos de fragmentos de piel y de costras revelaron la presencia de cuerpos de inclusión intracitoplasmáticas, envueltos por membrana y conteniendo partículas ovales, midiendo 270 x 130 nm, conteniendo núcleo o centro interno bicóncavo (forma de mancuernas). Partículas inmaduras (vacías) envueltas por membrana fueron observadas. También fueron analizados cuerpos de inclusión intracitoplasmáticos, electrodensos, conteniendo partículas virales brotando del material denso y amorfo. Fueron observadas inclusiones intranucleares fibrilares o “digitales” mostrando una estricción periódica y disposición en grupos en medio del material granular. Los núcleos estaban deformados con cromatina densamente condensada formando cuerpos de inclusiones electrodensas y amorfas. En la técnica de inmunocitoquímica la reacción antígeno-anticuerpo fue intensamente marcada por las partículas de oro coloidal realzando fuertemente las partículas virales.

PALABRAS CLAVE: Mixomatosis; Poxvirus; Conejo; Microscopía electrónica de transmisión.

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