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Study of Pseudorabies Virus, RC/79 Strain, Virulence Markers

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ABSTRACT. There are no antigenic differences between known strains of Aujeszky's Disease virus or the Pseudorabies virus (PV). The characterization of these viruses has been based on pathological and/or pathogenic aspects of the host. In this study, PV (RC/79 strain) virulence markers have been characterized based on their capacity to produce changes in monolayer cell cultures and by effects generated in experimentally infected rabbits. By analyzing the cytopathic effect induced on Vero cells by the virus, it was possible to visualize the formation of rounded-up cells at the beginning of the infection and later on the appearance of multinuclear or syncytial cells. Lysis plaques (4-5 mm) under agar had rounded, well defined borders. Studies carried out in cell cultures and experimental infections in rabbits showed the virulent nature of the RC/79 strain, for all cases studied. In this animal model intense pruritus was seen at the site of inoculation, with the appearance of a lesion exuding serum and blood from 6-24 h before death. For all cases, the mortality rate was 100%, even when smaller viral doses (102 PFU/ml) were used, which indicates that there was an absence of dose response. The rabbit marker was used to characterize the RC/79 strain of the PV as strongly virulent. This model could also be used in epidemiological studies to diagnose AD, differentiating between virulent and attenuated strains. It represents a useful tool that can be used to assess naturally attenuated virulent isolates that are potential vaccine candidates. Furthermore, the rabbit model may be useful to determine the degree of attenuation in viral strains that have been submitted to laboratory modifications.
Key words: Aujeszky's Disease Virus, Virulence Markers, Rabbit Inoculation.

RESUMEN. La caracterización de las cepas del virus de la enfermedad de Aujeszky o virus de la pseudorrabia (VPR) debe hacerse en base a los aspectos patológicos y/o patogénicos que origina en el huésped, debido a que no existen diferencias antigénicas entre las cepas . En este estudio se han podido caracterizar marcadores de virulencia VPR, cepa RC/79 por su capacidad para producir alteraciones en monocapas celulares y por los efectos generados en conejos experimentalmente infectados. En el análisis del efecto citopático inducido por el virus sobre células Vero, fue posible visualizar al inicio de la infección la formación de células redondeadas y posteriormente la aparición de células multinucleadas o sincicios. Las placas de lisis bajo agar (4-5 mm) tuvieron bordes redondeados y bien delimitados. Los estudios en cultivos celulares y la infección experimental en conejos indicaron el carácter virulento de esta cepa. Se puso en evidencia un intenso prurito en la zona de inoculación, con la aparición de una lesión rezumante serosanguinolenta entre las 6-24 h previas a la muerte. La tasa de mortalidad fue del 100%, aún trabajando con dosis menores de virus (102 UFP/ml) indicando ausencia de dosis-respuesta. El marcador "conejo" no sólo ha permitido caracterizar a la cepa RC/79 del virus de la pseudorrabia como fuertemente virulenta sino que además puede servir en estudios epidemiológicos facilitando el diagnóstico de la EA, permitiendo diferenciar cepas virulentas de las atenuadas, constituyéndose en una herramienta útil para controlar aislados virales naturalmente atenuados que puedan ser usados como agentes vacunales y/o para controlar el grado de atenuación de cepas virales que sufran modificaciones de laboratorio.
Palabras clave: Virus de la enfermedad de Aujeszky, Marcadores de Virulencia, Inoculación de conejos .

INTRODUCTION

The Pseudorabies virus (PV) is the etiological agent of Aujeszky's disease (AD). AD is characterized by producing marked respiratory and/or nervous changes, particularly in the natural host, the pig. Since PV was first isolated in the Republic of Argentina,[1] the incidence of the disease in the main pig producing areas of Argentina has increased.[6,12,20] In response to this data and in the absence of an official plan of campaign against AD, it is necessary to carry out epidemiological investigations.

Such epidemiological studies are an integral part of future control programs that will depend on the effective identification of isolates of Pseudorabies virus strains.

PV is a member of the Herpesviridae family, Alfaherpesvirinae sub-family. Unfortunately there are no antigenic differences between the known strains of the virus, hence their classification and characterization has been based on their physio-chemical properties and on pathological and pathogenic aspects in the host.[3]

From a practical point of view it is important to identify genetic markers that are related to virulence. These markers will then be used to distinguish between virulent and attenuated viral strains. For PV such markers have still not been defined. For example, a relationship between virulence and ***s*** (size of lysis plaques) or ***rct*** (ability to grow at high temperatures) has not been found.[21]

On the other hand, two models have been identified which distinguish between virulent and attenuated strains of PV. The first of these is known as the "rabbit marker", in which attenuated strains do not cause pruritus at the inoculation sites of rabbits[13] or in rats.[19] The second model involves the assessment of CE in cell cultures, such that attenuated strains cause rounding-up of cells, whereas virulent strains cause the formation of syncytial cells. These differences have been demonstrated in pig kidney cells,[11] calf kidney cells[22] and monkey kidney cells.[19]

It has also been established by immuno-chemical techniques that in attenuated PV strains, the viral Ag can mainly be seen in the cytoplasm of infected cells, while the virulent strains show nuclear fluorescence.[16,22]

The aim of this study is to characterize the virulence markers of the Pseudorabies virus, RC/79 strain. This strain was first isolated in Argentina in our laboratory by the experimental inoculation of rabbits and by *in vitro* infection of Vero cell monolayers.

MATERIALS AND METHODS

Viral Strain. Herpes suis type 1, RC/79 strain, was used. It was isolated in Río Cuarto in 1979 in chicken embryo fibroblast cultures. Isolates were taken from the central nervous system of pigs that were infected during an epidemic outbreak, and a titer of 1 x 103.5 DICC50/ml was detected.[1] The virus was passaged ten successive times in Vero cell cultures until a titer of 1 x 108 DICC50/ml was obtained. This viral stock was kept at -20ºC and was maintained in the laboratory for successive passages.

Cellular System. Vero cells from a line originally derived from kidney cells of the African green monkey (*Cercopithecus aethiops*) were used in all experiments. The cells were grown as monolayers on glass or plastic surfaces in Eagle-Earle's minimal essential medium (MEM), supplemented with 8% fetal bovine serum and 30 µg/ml of glutamine and antibiotics. Cells from two sources were used indiscriminately. One source, which had undergone between 105 and 171 passages, originated from Virology department in the Exact Sciences Faculty, (Facultad de Ciencias Exactas) in the University of Buenos Aires (UBA). The other source, strain 76, with 32-40 passages, was obtained from the ABAC (Asociación Banco Argentino de Células, The Argentine Cell Bank Association) in the city of Pergamino, province of Buenos Aires.

Rabbits. To study viral strain pathogenicity, 8 female New Zealand white rabbits (*Oryctolagus cuniculus*) were used. The animals weighed from 1.4 to 2.3 kg and were 1.5 to 2.5 months old. The animals were provided by the Central Animal House at the National University of Río Cuarto.

Determination of virulence markers by in vitro assays. To characterize the type of cytopathic effect (CE) produced by the RC/79 viral strain of AD, Vero cell monolayers were grown in 24 well plates or in 15 ml glass bottles. They were infected with a viral dilution, which produced an approximate rate of infection of 1:1. The cells were then incubated at 37 ºC. Viral damage was assessed by microscopic examination over a period of 3 days.

Characterization of the lysis plaques. In parallel, the type of lysis plaque generated by the virus strain was characterized. This was carried out by infecting four bottles as described above, to achieve a 1:1 infection ratio. To these bottles plaque medium (double concentration of MEM plus 2% methylcellulose in double distilled water) was added and the cultures were incubated for 4 days at 37 ºC. At the end of this time the lysis plaques were revealed following the method described by Dulbecco.[5] As a control, four bottles were left with non-infected cellular monolayers. These controls were subjected to the same procedure as the infected monolayers.

In vivo assays. Studies on pathogenicity in rabbits. Virulence of the RC/79 strain was determined in rabbits. 8 animals were inoculated with 1 ml of viral suspension in MEM supplemented with 2% inactivated fetal bovine serum. The suspension was inoculated subcutaneously, in the inferior dorsal flank.

To analyze the influence of viral dose on the clinical response, 3 animals were inoculated with a viral dose of 102 PFU/ml and the remaining 5 animals received 105 PFU/ml. Observation of the symptoms was carried out for as long as the test allowed, which was on average for no more than 120 h post infection (p.i.). All the animals were kept in the animal house, at ambient temperature and in individual cages.

RESULTS

Characterization of CE. Vero cell monolayers infected with PV-RC/79 were checked daily. The formation of groups of large, refractive rounded-up cells were observed at the beginning of the infection (16-24 h p.i.). Later on during the infection, these cells made up the focus of lysis with detachment of cells from the glass walls, revealing holes in the cell culture (Fig. 1-arrow a).

In parallel, between 24 and 36 h p.i., it was possible to

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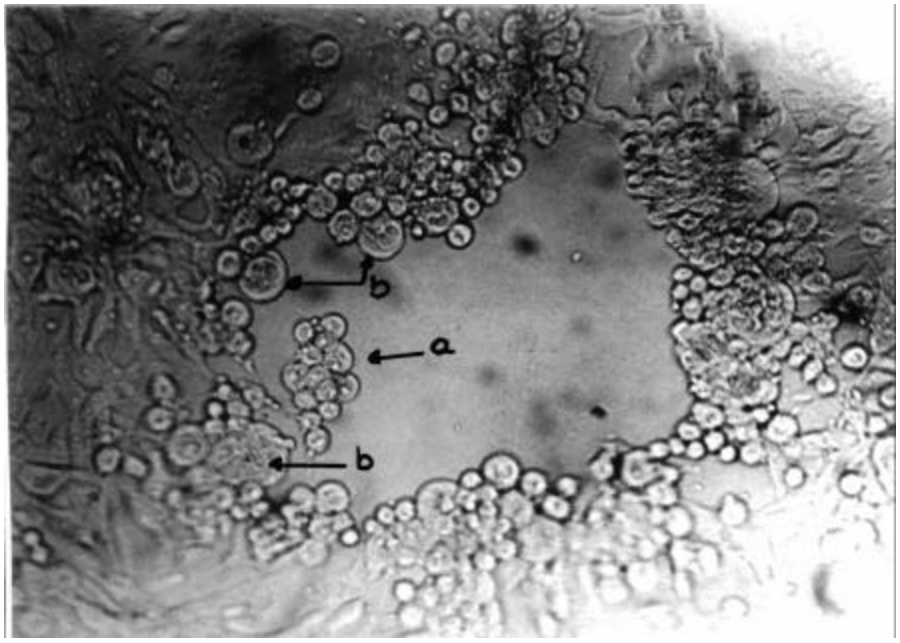

Fig. 1. Cytopathic effect of the Pseudorabies virus, RC/79 strain, on Vero cell monolayers at 16 - 36 hours p.i. Arrow 'a' indicates rounded-up cells and arrow 'B' indicates syncytium, resulting from cell fusion. (x 20).

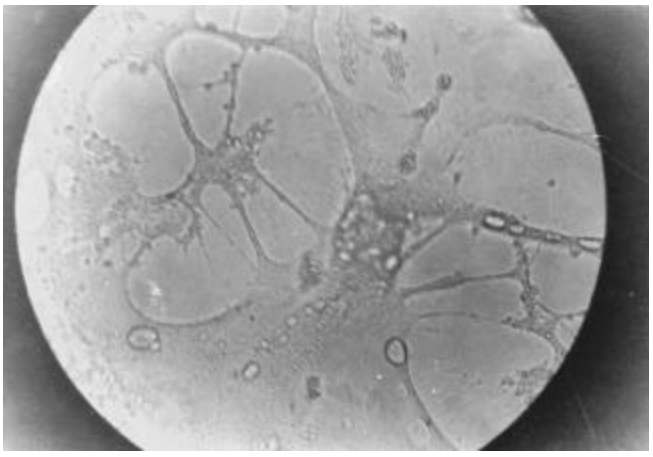

Fig. 2. Vero cell monolayers showing cytopathic effects caused by the Pseudorabies virus, RC/79 strain, 24-36 h

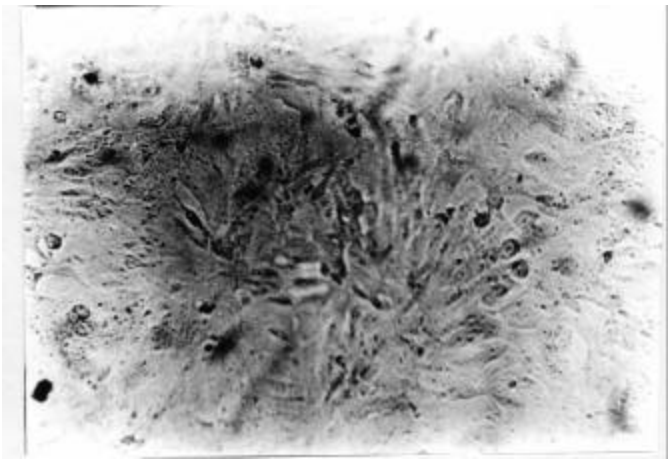

Fig. 3: Uninfected control Vero cell monolayers. (x 40).

see the appearance of multinuclear or syncytial cells produced as a consequence of cellular fusion (Fig. 1, arrow b).

A certain number of cells that did not undergo lysis remained united to others by cytoplasmic connections and had the appearance of asteroid formations (Fig. 2).

In Fig. 3 the control non-infected Vero cell monolayers can be seen.

Characterization of lysis plaques. Fig. 4 shows the lysis plaques caused by PV-RC/79 under agar. These were seen after 24 h p.i., and reached their greatest number and size at day three p.i.

It can be observed that the plaques are bright, with regular borders and have a diameter of 4-5 mm.

Tests of pathogenicity in rabbits. Table 1 shows the results obtained from inoculation tests carried out in rabbits. Animals were infected with different concentrations of virus, which varied from 102 to 105 PFU/ml. Changes in the clinical response to these different viral concentrations were not found, since all the animals died.

In each of the infected rabbits, periods of nervous excitement were observed during which they moved violently within their cages, followed by periods of tranquillity. Also, pruritus was observed on each animal at the inoculation site, which occurred between 6 and 24 h before death. As a consequence of the pruritus the animals savagely bit the area around the injection site, and this auto-mutilation resulted in the area around the site being left completely hairless, with cutaneous abrasions that exuded blood and serum (Fig. 5a). In the same figure it is possible to see differences in the texture of the animal's fur. Before viral in-

Fig. 4. Lysis plaques caused by the Pseudorabies virus, RC/79 strain, revealed by crystal violet staining at 72 h p.i.

oculation (Fig 5b), the fur was flat and silky while after infection the fur became rough and bristly.

The rabbits that received smaller viral doses (102 PFU) took longer to die, on average 120 h p.i.. The time of death was 1.9 times greater than for the 5 animals that received 105 PFU that died between 54 and 66 h p.i.

DISCUSSION

Studies carried out by A. Sabin in 1934, on the Herpes simplex virus (HSV) and the Pseudorabies virus in animals, have revealed that these two viral types are related.[15] The reasoning behind this conclusion was based on the fact that both viruses are pantotrophic, neuro-invasive, produce similar intracellular inclusions and have a similar range of hosts. Furthermore, the epidemiological patterns of HSV and PV are also similar.

Later a more detailed study was carried out that made comparisons between both viruses.[9] In this article cell cultures infected with PV were described. There was initially the appearance of few areas of focal necrosis and rounded-up cells, and a few h later (8-10 h) it was possible to see the formation of syncytial cells in the monolayer, where the cell walls disappeared and consisted of a large multinucleated syncytial mass. Based on studies carried out by T. Tokumaru in 1957, these two different classes of CE were attributed to genetic heterogeneity within the viral population.[19] This author demonstrated that the difference in the characteristics of CE was due to strain virulence. It was concluded that in general the attenuated strains caused rounding-up of cells while the virulent strains initially caused cellular rounding-up but also caused the formation of syncisia.

In this study, characterization of the PV-RC/79 strain was based on cytopathogenic effects on Vero cell monolayers, and it was possible to observe a progression in CE. This progression started with the rounding-up of cells and lead to the formation of syncisia and cellular detachment as a consequence of cellular lysis (Fig. 1a, b and 2).

With the aim of demonstrating differences between lysis plaques induced by virulent and attenuated strains, Tokumaru carried out single step growth curves for each viral type, using immune sera.[19] It was shown that the viral strains that generate clinical symptoms of Pseudorabies in rabbits and rats, and that induced formation of syncisius in cellular monolayers, have a tendency to generate large or "L" lysis plaques, however no specific values were given. Sabin on the other hand, described plaques produced by virulent strains that had diameters of between 5-10 mm.[15]

In the characterization of the type of lysis plaques generated by PV-RC/79, it was observed that the sizes of these plaques (4-5 mm) correspond to those described for virulent strains (Fig. 4). This was reaffirmed by the results from the type of CE induced by the virulent viral strain, as well as by its progression toward the formation of syncisius.

From another point of view, the virulent character of PV-RC/79 was revealed by results obtained from the rabbit

Fig. 5. a, White rabbit subcutaneously inoculated with the Pseudorabies virus, RC/79 strain, showing hair loss and skin abrasion at the inoculation site, 36 h p.i. b, Appearance of the rabbit flank before viral inoculation.

Table 1: Determination of virulence of the Pseudorabies virus, RC/79 strain, by clinical response and mean time of death, in white rabbits inoculated subcutaneously.

RC/79 strain Dilution:	Nº of inoculated rabbits	Nº of deaths	% mortality	Mean time of death@	Range	Pruritus
105 PFU	5	5	100	62	54 - 66	Yes
102 PFU	3	3	100	120	114 - 126	Yes

@ Hours post inoculation (p.i.)

infection tests. Subcutaneous injection of rabbits with a suspension of animal tissue suspected of containing the AD virus, was described in an empirical way by the first researchers into this disease.[2,8,10,18] These authors reported elevated animal receptiveness to the AD virus, with manifestations of characteristic pruritus and the appearance of an oozing lesion in the flank behind the inoculation site.

In an attempt to characterize the Pseudorabies virus Platt et al worked with 11 virulent North American strains and 2 attenuated European strains.[14] They based their analysis on thermic sensitivity of the strains and on virulence in rabbits. The viral strains were subsequently categorized into 3 groups according to their thermostability (those resistance to heat, moderately resistant to heat and thermo sensitive) and into 3 groups according to their ability to clinically infect rabbits, their ability to produce pruritus and the time before the animal's death. In this final category the strains were considered to belong to type I when the rate of clinical infection was less than 25%, from type II when no pruritus was produced and the average time of death (ATD) was more than 78 h p.i., and from type III or strongly virulent, when pruritus was produced and the ATD was less than 66 h p.i. These authors demonstrated that the kinetics of thermic inactivation and the parameters used to compare PV in rabbits, could be used in conjunction to effectively differentiate between attenuated strains of PV isolated in the field and would serve as an aid to epidemiological investigations.

Among the viruses studied the attenuated strains K and BUK have been found. These strains have been shown to be highly resistant to heat and with respect to their ability to clinically infect rabbits, correspond to categories I and II, respectively. These results contrast with those for the virulent Be strain (among other virulent strains studied) that produced a 100% mortality rate in rabbits and were shown to be thermo-sensitive. In tests carried out by Platt et al, it was shown that a strongly virulent strain generates all the clinical symptoms described in rabbits and is also heat sensitive.

In this study, it has been demonstrated that the RC/79 strain generates 100% mortality in rabbits. This finding confirms that it is a virulent strain, as suspected since it was first isolated following its history of generating 80% mortality in piglets.[1] The virulent character of the RC/79 strain is also supported by the absence of a dose response. Animals were given viral inoculation of between 102 and 105 PFU, with lower viral doses only the average time of death was modified, and in all cases the fatality in rabbits was 100% (Table 1).

In a previous work our group has demonstrated that the PV-RC/79 strain is sensitive to heat and trypsin.[17] By comparing this data with those obtained in this rabbit infection test, it is possible to see that the RC/79 strain is strongly virulent, which is in agreement with the results obtained by Platt et al.[14]

The "rabbit marker" has an important role in epidemiological studies. Its use would help in the diagnosis of Pseudorabies by distinguishing between virulent and attenuated strains. Furthermore this test represents a useful tool when screening for naturally attenuated viral isolates that may be used as vaccines and/or it may be used to control the degree of attenuation of viral strains that are modified in the laboratory.

It must be pointed out that some authors have described strains of the AD virus that cause high mortality in the field but do not produce pruritus following intracerebral or subcutaneous inoculation, at least following 2 or 3 passages.[4,7] This observation reaffirms the fact that the RC/79 strain, by generating the symptoms of pruritus and death with only one subcutaneous passage in rabbits and with lower viral doses (102 PFU), is strongly virulent.

With this experience it has been possible to characterize the PV-RC/79 strain with virulence markers and in parallel we have adjusted a valuable diagnostic tool consisting of the experimental inoculation of rabbits with clinical samples. This technique is available in our laboratory and it is possible to apply it to AD control or eradication programs.

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REFERENCES

1. Ambrogi, A., J. Giraudo, J. Busso, O. Bianco, E. Bagnat, M. Segura de Aramburu, B. Ramos and F. Ceriatti. 1981. Primer diagnóstico de la enfermedad de Aujeszky en cerdos en la República Argentina . Gat. Vet. Buenos Aires. Tomo XLII, Nº 357:58-64.
2. Aujeszky A. 1902. Ueber eine neue Infektions Krankheit bei Haustieren. Zentr. Bakt. Parasitkde Abt. I. Orig. 32:353-357.
3. Baskerville, A., J. B. McFerran and C. Dow. 1973. Aujeszky's Disease in Pigs. Vet. Bull. 43:465-480.
4. Christov, S., N. Pavlov, I. Karadjov, D. Beltchev and I. Deltchev. 1966. Enzooties d'encéphalomyélite aigu chez des veaux nouveanu-nés causées par le virus de la maladie d'Aujeszky. Bull. Off. Int. Epizoot. 65:1247-1264.
5. Dulbecco, R. 1962. Production of plaques in monolayer tissue culture by single particles of an animal virus. Proc. Natl. Acad. Sci. USA 38:747-752.
6. Echeverria, M. G., E. O. Nosetto, E. J. Gimeno, C. M. Galosi, N. B. Pereyra, R. D. Fonrouge and M. E. Etcheverrigaray. 1989. Pseudorrabia (enfermedad de Aujeszky) en cerdos: estudios seroepidemiológicos. In. Memorias. V Reunión Anual de la Asociación Argentina de Veterinaria de Laboratorio de Diagnóstico, Río Cuarto, Córdoba. Argentina.
7. Gordon, W. A. and D. Luke. 1955. An outbreak of Aujeszky's disease in swine with heavy mortality in piglets, illness in sows and death in utero. Vet. Rec. 67:591-597.
8. Hutyra, F. 1910. Beitrag zur Atiologie der infektiösen Bulbärparalyse. Berl. Munch. Tierärztl. Wschr. 26:149-151.
9. Kaplan, A. S. and A. E. Vatter. 1959. A comparison of Herpes simplex and pseudorabies viruses. Virology 7:394-407.
10. Köves, J. and G. Hirt. 1934. Über die Aujeszkysche Krankheit der Schweine. Arch wiss. prakt. Tierheilk. 68:1-23.
11. Lomniezi, B. 1974. Biological properties of Aujeszky's disease (Pseudorabies) virus strains with special regard to interferon production and interferon sensitivity. Arch. Ges. Virusforsch. 44:205-214.
12. Pereyra, M. O., N. B. Pereyra and F. D. Cané. 1989. Situación de la enfermedad de Aujeszky en Chañar Ladeado. V Reunión Anual de la Asociación Argentina de Veterinaria de Laboratorio Diagnóstico. Río Cuarto. Córdoba. Argentina.
13. Petrović, M. M. 1967. Comparative study on biological properties of some field strains in a vaccine of Aujeszky's disease virus (in Srbocroatian). Acta Vet. (Beogr.) 17:335-343.
14. Platt, K. B., C. Maré and P. Hinz. 1979. Differentiation of vaccine strains and field isolates of Pseudorabies (Aujeszky's disease) virus: Thermal sensitivity and rabbit virulence markers. Arch. Virol. 60:13-23.
15. Sabin, A. 1934. The immunological relationships of pseudorabies (infectious bulbar paralysis, mad itch). Brit. J. Exptl. Pathol. 15:372-380.
16. Sabini L. I., F. S. Ceriatti, G. Bagnis, S. M. Zanon, V. Martin, M. Rovera and B. A. Ramos. 1997. Técnicas diagnósticas de referencia para el análisis de marcadores de virulencia del virus pseudorrabia, cepa RC/79: ensayos preliminares. Archivos de Medicina Veterinaria, Facultad de Cs. Veterinarias. Universidad Austral de Chile 29:251-260.
17. Sabini, L. I., F. S. Ceriatti, B. A. Ramos and S. M. Zanon. 1996. Sensibilidad al calor y la tripsina del virus Herpes suis cepa RC/79". Archivos de Medicina Veterinaria. Facultad de Cs. Veterinarias. Universidad Austral de Chile 28:159-163.
18. Shope, R. E. 1934. A contagious disease in swine. Science 80:102-103.
19. Tokumaru, T. 1957. Pseudorabies virus in tissue culture: differentiation of two distinct strains of virus by cytopathogenic pattern induced. Proc. Soc. Exp. Biol. (New York) 96:55-60.
20. Zanon S., S. Bettera, L. Sabini, S. Ceriatti, B. A. Ramos and A. Ambrogi. Abril-Junio 1991.Relevamiento seroepidemiológico para el virus de la enfermedad de Aujeszky en piaras de la región de Río Cuarto (Pcia. de Córdoba, Argentina). Revista Veterinaria de Uruguay 27:14-16.
21. Zuffa, A. and K. Grígelová. 1966. Immunisierung gegen die Aujeskysche Krankheit. II. Studium der Zytopathogenität und Plaquenmorphologie verschiedener Virusstämme bezüglich ihrer Virulenz für Schweine. Arch. Exp. Med. 20:127-140.
22. Zuffa, A., J. Matis and V. Pleva. 1968. Studies on several strains of Pseudorabies (Aujeszky) virus showing different virulence by means of the immuno and acridine orange fluorescence. Arch. Ges. Virusforsch. 24:396-405.