Research in Veterinary Science 95 (2013) 709–716

Contents lists available at SciVerse ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Systemic infection induced by intranasal inoculation of *Bovine herpesvirus* 1.1 in pregnant and non-pregnant rabbits

A.R. Valera [a], N.A. Fuentealba [a,e,f], C.N. Zanuzzi [b,c,e], S.G. Corva [d], M.R. Pecoraro [a], C.G. Barbeito [b,c,e], C.M. Galosi [a,f,*]

[a] Department of Virology, Faculty of Veterinary Sciences, National University of La Plata, 60 & 118, P.O. Box 296, 1900 La Plata, Buenos Aires, Argentina
[b] Department of Histology and Embryology, Faculty of Veterinary Sciences, National University of La Plata, 60 & 118, P.O. Box 296, 1900 La Plata, Buenos Aires, Argentina
[c] Department of Pathology, Faculty of Veterinary Sciences, National University of La Plata, 60 & 118, P.O. Box 296, 1900 La Plata, Buenos Aires, Argentina
[d] Department of Epidemiology, Faculty of Veterinary Sciences, National University of La Plata, 60 & 118, P.O. Box 296, 1900 La Plata, Buenos Aires, Argentina
[e] National Research Council (CCT-CONICET-La Plata), Argentina
[f] Scientific Research Commission of Buenos Aires Province (CIC-PBA), Argentina

ARTICLE INFO

Article history:
Received 8 October 2012
Accepted 25 March 2013

Keywords:
Abortion
Bovine herpesvirus 1.1
Intranasal inoculation
Rabbit model
Systemic infection

ABSTRACT

Bovine herpesvirus (BoHV) type 1.1 (BoHV-1.1) causes repeated outbreaks of upper respiratory disease and abortion in cattle. The systemic effects of BoHV-1.1 in rabbits, using intranasal inoculation are reported. Female rabbits were divided into four groups and inoculated with the virus 10 days before mating, and at 15 or 22 days of pregnancy. Studies of the clinical signs, antibody production, virus isolation, and DNA detection as well as histological and immunohistochemical studies were carried out on lungs, kidneys, spleen, placentas, uteri and foetal tissues. All virus-inoculated animals developed respiratory clinical signs and a humoral response. BoHV-1.1 was isolated from nasal swabs and plasma rich in leukocytes, and viral DNA was detected in blood, dead foetuses and placentas. Histopathological lesions were found in the respiratory tract and some placentas and foetuses were immunohistochemically positive. Intranasal inoculation might be useful to study the systemic effects of BoHV-1.1 infection in the rabbit model.

1. Introduction

Bovine herpesvirus (BoHV) type 1.1 (BoHV-1.1) is responsible for repeated outbreaks of respiratory disease (infectious bovine rhinotracheitis) and abortion in cattle, usually at the fourth month of pregnancy. Abortion induced by BoHV-1.1 is one of the main viral causes of reproductive failure in bovine production (Smith, 1997). BoHV-1.1 enters the host through the mucous membrane of the upper respiratory tract and is preferentially transmitted by direct nose-to-nose contact. After replication in the mucosal epithelium, BoHV-1.1 infects sensory neurons of the peripheral nervous system and animals become latent carriers. BoHV-1 spreads in the host during a viraemic phase and gains access to a broader range of tissues and organs to cause many disorders, including abortion. However, it is still unknown how BoHV-1.1 gains access to the placenta and foetus. The presence of viral lesions in foetal livers suggests that haematogenous dissemination may occur via the umbilical vein (Muylkens et al., 2007). The rabbit model has been used to study the effects of different species of BoHV, such as BoHV-5 (Rock et al., 1987; Meyer et al., 1996; Lee et al., 1999; Silva et al., 1999; Spilki et al., 2002), BoHV-4 (Osorio et al., 1982; Naeem et al., 1990, 1991) and BoHV-1.1 (Smith, 1978; Honda et al., 1991). So far, the rabbit model has been the best to study upper respiratory disease, conjunctivitis and meningoencephalitis induced by BoHV-1.1 (Lupton et al., 1980; Chowdhury et al., 1997) inoculated by different routes. Kelly (1977) induced the infection by inoculating adult and newborn rabbits with BoHV-1, by the intra-peritoneal, intra-conjunctival and intra-cerebral routes. Rock and Reed (1982) infected adult rabbits with BoHV-1, using intra-conjunctival inoculation, and activated the infection after treatment with corticosteroids. In addition, Honda et al. (1991) inoculated rabbits by the intra-tracheal and intravenous routes and analysed the antibody response, but failed to reproduce respiratory clinical signs. Although different routes of inoculation were reported to experimentally reproduce many aspects of the natural infection in these studies, none used intranasal (i.n.) inoculation, the natural route of entry of BoHV.

After i.n inoculation of the virus, Brown and Field (1990) reproduced the neurological disease induced by BoHV-5; Chowdhury

* Corresponding author at: Department of Virology, Faculty of Veterinary Sciences, National University of La Plata, 60 & 118, P.O. Box 296, 1900 La Plata, Buenos Aires, Argentina. Tel./fax: +54 221 4824956.
E-mail addresses: cmgalosi@fcv.unlp.edu.ar, cmgalosi@yahoo.com (C.M. Galosi).

0034-5288/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.rvsc.2013.03.017

et al. (1997) confirmed the neuroinvasion of BoHV-5, and Silva et al. (1999) described relevant aspects of the meningoencephalitis induced by Brazilian isolates of BoHV-5 in rabbits.

Abortion induced by other members of the subfamily *Alphaherpesvirinae* of veterinary interest has been largely studied in experimental animal models (Babic et al., 1994; Awan et al., 1995). However, the rabbit model of abortion induced by BoHV-1 has been mentioned as an accidental finding in some studies using inoculation routes other than i.n (Lupton et al., 1980; Kelly, 1977). Since there are no recent data regarding BoHV-1 infection in pregnant rabbits, the goal of this work was to study the systemic effects of BoHV-1.1 in female rabbits after i.n inoculation. In addition, we aimed to determine whether the i.n route could be used to investigate reproductive failures induced by BoHV-1.1 infection in pregnant rabbits.

2. Materials and methods

2.1. Virus and cell cultures

The Los Angeles (LA) strain obtained from The National Institute of Agricultural Technology (INTA), Castelar, Argentina, was used for the experimental infection. Virus stocks were prepared in Madin-Darby bovine kidney (MDBK) cells routinely grown in Eagle's Minimal Essential Medium (EMEM) containing 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 100 IU/ml nystatin and supplemented with 10% foetal calf serum (FCS). Cells were maintained in EMEM with 2% FCS (M-EMEM). A confluent monolayer of cells was infected and incubated with M-EMEM. When extensive cytopathic effect (CPE) was observed, the cells were frozen at −70 °C. After three cycles of freezing and thawing, the cell suspension was centrifuged at 10,000*g* for 30 min to remove cell debris and the virus suspension was fractionated in small volumes, quantified by the standard Reed and Muench method (Reed and Muench, 1938), and stored at −70 °C until used.

2.2. Animals and experimental design

Animal handling and all experimental procedures were carried out in compliance with the recommendations of the "Guide for the Care and Use of Laboratory Animals of the National Research Council" (Academy Press, 1996, Washington, USA, http://dels.nas.edu/ilar_n/ilarhome/) and supervised by the Institutional Committee for Care and Use of Laboratory Animals (Faculty of Veterinary Sciences, National University of La Plata, Buenos Aires, Argentina). Twenty-three 5-month-old New Zealand white pregnant female rabbits and seven non-pregnant ones were used. Rabbits were maintained in separate cages in conventional animal facilities with food and water *ad libitum*. Gestation was confirmed by abdominal palpation at 10–12 days post-mating (dpm) since it was not possible to perform ultrasound. The animals were divided according to the time of virus inoculation into four groups (Groups 1–4), each consisting of three Sub-groups (a–c) formed by mock (b) or virus-inoculated (a and c) pregnant (b) or non-pregnant (c) rabbits (Table 1).

The animals of Sub-groups "a" and "c" were infected with 500 μl of the infected MDBK cells supernatant ($10^{6,5}$ $CCID_{50}$/50 μl) as previously described (Valera et al., 2008). After inoculation, the remaining inoculums were quantified again to re-confirm the dose inoculated. Rabbits from Sub-group "b" received 500 μl of uninfected MDBK cell supernatant. Each Group was taken as an individual experiment, and all the assays were developed sequentially according to the availability of the facilities.

Table 1
Experimental design. Number of animals per Group and Sub-group used in the experiment.

Sub-groups	Groups			
	1	2	3	4
a	4[#]	4[*]	4[*]	3[*]
b	2[*]	2[*]	2[*]	2[*]
c	2	2	2	1

Group 1: inoculated at 10–12 days before mating.
Group 2: inoculated at ~15 days of pregnancy.
Group 3: inoculated at ~22 days of pregnancy.
Group 4: inoculated at ~15 days of pregnancy; sacrificed 5 days post-infection.
a: mated and virus-inoculated.
b: mated and mock-inoculated.
c: non-mated and virus-inoculated.
[*] Pregnancy confirmed at 10–12 days post-mating.
[#] Pregnancy diagnosis negative at 10–12 days post-mating.

2.3. Clinical assessment and collection of samples

After inoculation, rabbits were examined daily for clinical signs of respiratory disease and rectal temperatures were taken. Serum samples were obtained to determine BoHV-1.1 antibody response 10–90 days post-inoculation (pi). In addition, heparinised blood samples (20 IU/ml) were collected at 48 and 72 h pi from animals of Sub-groups "a" and "c" of Groups 1–3. Animals from Sub-groups "a" and "c" of Group 4 were alternatively bled from each marginal ear vein, every ~12 h from ~24 h pi until ~96 h pi. Animals from Group 4 were used to determine the period of viraemia to maximise viral recovery. Blood was immediately centrifuged at 1,000*g* for 15 min, the buffy coat was mixed for 1 min with erythrocyte-lysis buffer (0.85% NH_4Cl, 0.017 M Tris, pH 7.4) and centrifuged at 1,000*g*. The supernatant was removed and the pellet suspended in the original supernatant recovered in the first centrifugation. The resulting plasma, rich in leukocytes, was used immediately for virus isolation and processed using a commercial kit (Wizard Genomic DNA Purification Kit, Promega, Madison, USA) for DNA detection by polymerase chain reaction (PCR). Nasal swabs were collected daily from 24 h pi until the absence of respiratory signs. These samples were immediately immersed in 1 ml of M-EMEM and vigorously shaken for 15 s before the determination of the amount of virus by infectivity titration. Samples from lungs, liver, kidneys and spleen from foetuses and stillborn rabbits from Groups 2 and 3 were also collected. Placentas were also collected whenever possible. In addition, samples from the uteri, lungs, kidneys and spleen were also collected from dead or sacrificed females during pregnancy and from post-partum rabbits. All samples were submitted for histological and virological studies (virus isolation and PCR). Two rabbits of Sub-group "a" of Group 4 were sacrificed 5 days pi (dpi) and samples similar to the ones mentioned above were collected. One pregnant rabbit used as negative control (Sub-group "b") was killed at the same time as virus-inoculated rabbits from Sub-group "a", whereas the other negative control rabbit continued pregnancy until normal parturition. For virological studies, all tissue samples were processed by preparing a 10% (wt/vol) homogenised suspension in M-EMEM, clarified by centrifugation at 6,000*g* for 20 min, and the supernatants used for virus isolation. For DNA detection, samples were processed according to the manufacturer's instructions (Wizard Genomic DNA Purification Kit, Promega, Madison, USA). Samples immediately fixed in 10% formalin were used for histological studies.

2.4. Infectivity titration

Infectivity titration was performed on MDBK cells by inoculation with serial 10-fold dilutions in M-EMEM of nasal swab

samples. The titres were calculated by the Reed and Muench method at 96 h pi and expressed as $CCID_{50}$/ 50 μl.

Coverslip cultures inoculated with swab fluids and incubated for 24 h were fixed and analysed by standard direct immunofluorescence using a commercial anti-BoHV-1.1 serum fluorescent conjugate (VMRD, Inc. WA, USA).

2.5. Detection of antibodies and polymerase chain reaction (PCR)

Detection of antibodies was determined by conventional indirect ELISA previously standardized (Valera et al., 1999). The titres were expressed as reciprocal of the highest dilution over the cut off value equivalent to two average values of negative serum. PCR was conducted using a pair of specific oligonucleotide primers derived from the BoHV-1.1 thymidine kinase genomic region according to the methodology described by Alegre et al. (2001).

2.6. Virus isolation

Each sample was inoculated onto preformed MDBK cells and, after 2 h of incubation at 37 °C, the cells were overlaid with M-EMEM and monitored for CPE for one week. Samples without evidence of CPE were passaged before being considered negative.

2.7. Histology and immunohistochemistry

The samples were prepared by using standard histological techniques. Tissues were paraffin-embedded and 5-μm sections were stained with haematoxylin and eosin (HE). Some sections were processed for immunohistochemistry (IHC) and incubated with the primary anti gD-monoclonal antibody (Centre of Animal Virology – CEVAN – Buenos Aires, Argentina) diluted 1:100 in phosphate-buffered saline (PBS). Slides were incubated at 37 °C for 60 min with the primary antibody and then rinsed in PBS three times and incubated with anti-mouse EnVision® detection system + HRP (DakoCytomation, California, USA) at room temperature for 30 min. Liquid 3,3-diaminobenzidine tetrahydrochloride was used as chromogen (DakoCytomation), and Hill's haematoxylin was used for counterstaining.

2.8. Statistical analysis

Virus titres were evaluated by ANOVA, in a nested design (Group/Female) with repeated measures (DPI), using Stata software. For the dataset with missing values (animals without clinical signs between 6 and 7 dpi), ten different multiple imputed datasets were generated based on the missing at random (MAR) technique and analysed by linear mixed modelling using the "ice" and "mim" packages for the Stata software .

3. Results

3.1. Clinical assessment

The temperature of virus-inoculated animals ranged within normal values (38.6–40 °C). In the four Groups, rabbits from Sub-group "a" showed intense respiratory clinical signs which consisted of severe serous nasal discharge during the first two days pi, followed by seromucinous discharge and laboured breathing; clinical signs disappeared between 6–7 dpi. In the four Groups, rabbits from Sub-group "c" showed similar clinical signs and time recovery.

Pregnancy diagnosis by palpation at 10 dpm was negative in all animals from Sub-group "a" of Group 1. These animals were kept under observation for the following 25 days, but none of them reached parturition. Two rabbits from Sub-group "a" of Group 2 died during parturition or four days before. One rabbit from Sub-group "a" of Group 3 died on day four pi, showing clinical signs of acute pneumonia. The surviving newborn female rabbits of Group 3 were screened for antibodies by ELISA one month after birth. The individual findings are detailed in Table 2. One rabbit from Sub-group "a" of Group 4 gave birth to no live neonates. In the four Groups, rabbits from Sub-group "b" showed no clinical signs and those not sacrificed at this time of the experiment reached normal parturition.

Table 2
Individual results obtained from pregnant rabbits inoculated at ~15 days of pregnancy (Groups 2 and 4) and ~22 days of pregnancy (Group 3) by intranasal inoculation with 500 μl of BoHV-1.1 Los Angeles strain ($10^{6,5}$ $CCID_{50}$/50 μl).

Group and animal	Time of inoculation (dpm)	Parturition	Pregnancy results	ELISA test (£)
G2-a1	14	Normal	1 neonatal death* 2 rabbits born dead*	ND
G2-a2	13	Death during parturition*,#	3 rabbits born dead* 8 live foetuses	ND
G2-a3	15	Death 4 days before parturition*,#	2 foetuses*	ND
G2-a4	15	Normal	2 hypokinetic neonates* 2 hypokinetic neonates dead by crushing	ND
G3-a1	22	Normal	1 rabbits born dead* 2 live newborns	1/100
G3-a2	21	Death 6–7 days before parturition*,#	4 dead foetuses*	ND
G3-a3	24	Normal	5 live newborns	(–) to 1/100
G3-a4	22	Normal	1 newborn dead crushed 5 live newborns 1 hypokinetic newborn*	Doubtful
G4-a1	15	Sacrificed 5 dpi	4 dead foetuses 2 live foetuses	ND
G4-a2	15	Sacrificed 5 dpi	3 dead foetuses* 2 live foetuses*	ND
G4-a3	14	Negative	ND	ND

G: Group.
a1–2–4: individual animal.
dpm: days post-mating.
dpi: days post-infection.
* samples and # placentas analysed by virus isolation, PCR and histological studies.
£ ELISA results from a pool of sera from one-month-old rabbits.
ND: not determined.

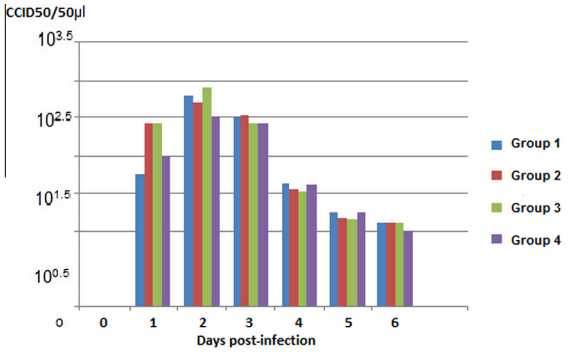

Fig. 1. Virus recovered during the first 6 dpi from nasal swabs of rabbits inoculated with 500 µl of the virus supernatant ($10^{6,5}$ $CCID_{50}$/50 µl). The titres were calculated at 96 h pi and expressed as log10 CCID50/sample. Bars represent the average titre obtained from all nasal swabs individually processed. Virus titres obtained on different days for the same animal were significantly different ($p < 0.05$). No significant differences were found ($p = 0.99$) between the four Groups of virus-inoculated animals.

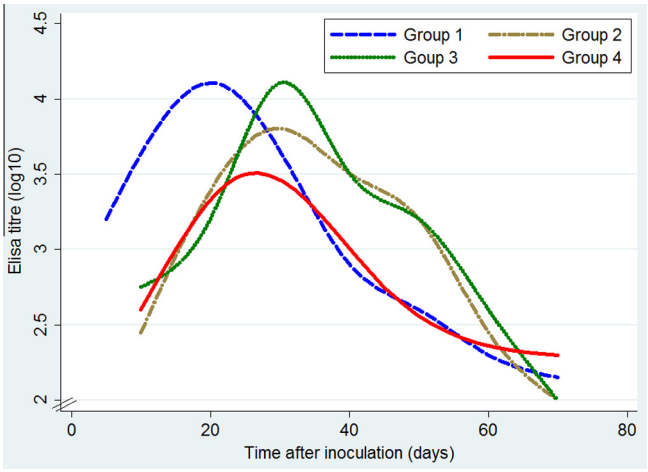

Fig. 2. Spline median graph of antibody response measured by ELISA. Results obtained from sera from pregnant rabbits and positive controls of Group 1 (inoculated at ~10 days prior to mating), Group 2 and 4 (inoculated at ~15 days of pregnancy) and Group 3 (inoculated at ~22 days of pregnancy) are shown. The antibody titres obtained at different days for the same animal were significantly different ($p < 0.05$). No significant differences ($p = 0.99$) were found between the four Groups of infected animals.

3.2. Infectivity titration

The virus was recovered from nasal swabs for at least 5–6 dpi, with an average peak of $10^{2.7}$ $CCID_{50}$/50 µl on the second dpi, when most of the clinical signs were observed.

Virus titres of the same animal measured on different days were significantly different ($p < 0.05$). No significant differences were found ($p = 0.99$) between Sub-groups "a" of the four Groups (Fig. 1). The immunofluorescence test confirmed that the isolated virus was BoHV-1.1. However, the virus was not recovered from any of the swabs of animals from Sub-groups "b" of the four Groups.

3.3. Antibodies detection, virus isolation and PCR

Antibodies to BoHV-1.1 were detected in sera at 10 dpi from virus-inoculated rabbits (Sub-groups "a" and "c") of all Groups. Antibody titre levels increased in surviving animals between 30–35 dpi, decreased between 70–80 dpi, and then became undetectable at 90 dpi. Antibody titres of the same animal measured on different days were significantly different ($p < 0.05$) (Fig. 2), whereas those between Sub-groups "a" of the four groups were not significantly different ($p = 0.99$). Virus isolation and PCR in plasma rich in leukocytes from rabbits from Sub-groups "a" and "c" of Groups 1–3 were negative, whereas those of Group 4 were positive at ~60 and ~72 h pi. The individual findings are detailed in Table 3. The lungs of rabbits of Group 4 were positive to virus isolation and PCR. No samples from dead foetuses born to females of Groups 2 and 3 were positive for virus isolation or PCR. Liver samples of two dead foetuses from two rabbits from Sub-group "a" of Group 4 were positive for PCR. No virus was isolated from placentas recovered from two rabbits from Sub-group "a" of Group 2 and one rabbit of Group 3. Some placenta samples from rabbits of Sub-group "a" of Groups 2 and 3 and from dead foetuses of rabbits of the same group of Group 4 were positive by PCR.

3.4. Histology and immunohistochemistry

The newborn rabbits of one rabbit from Sub-group "a" of Group 2 died within 24 h and showed areas of atelectasis in the lungs and haemorrhages in the liver, but no lesions in the kidneys. The lungs, kidneys and liver of the dead offspring from rabbits of Sub-group "a" of Group 2 showed autolytic changes. IHC was negative in all these samples.

The lesions found in the lungs of one rabbit from Sub-group "a" of Group 2 that died during parturition were consistent with suppurative bronchopneumonia, although no intranuclear inclusion bodies (IB) were observed.

The liver showed large areas of cellular degenerative fatty changes, coagulative necrosis, mononuclear infiltrate mainly with macrophages and lymphocytes, and sinusoidal dilatation. The kidneys showed areas of glomerular and intertubular haemorrhage, hyalinization and the presence of bacteria in the glomeruli. No lesions were found in the uteri. IHC was negative in the lungs, liver, kidneys, adrenal glands and uteri. All dead newborns that showed severe autolytic changes were excluded from the analysis.

One rabbit from Sub-group "a" of Group 2 died four days before parturition date. The lungs of this animal were atelectasic with an interstitial inflammatory response consisting of infiltration by macrophages and neutrophils. In addition, shrinking and necrosis of individual hepatocytes and a few inflammatory cells (mostly mononuclear cells and a few eosinophils) were observed in portal spaces. The kidneys and uteri of these rabbit showed no histopathological changes. Two foetuses of one female of this Sub-group and Group, two hypokinetic newborns of another virus-inoculated female of Group 2 and the dead newborn of one virus-inoculated female of Group 3 showed only fatty degenerative changes in the

Table 3
Individual results of virus isolation (VI) and PCR at different times post-infection from blood of pregnant (Sub-group a) and non-pregnant (Sub-group c) rabbits inoculated at ~10 days prior to mating (Group 1), ~15 days of pregnancy (Groups 2 and 4) and ~22 days of pregnancy (Group 3) by intranasal inoculation with 500 µl of BoHV-1.1 Los Angeles strain ($10^{6,5}$ $CCID_{50}$/50 µl).

Groups	Hours post-infection									
	~24–36		~48		~60		~72		~84–96	
	VI	PCR	VI	PCR	VI	PCR	VI	PCR	VI	PCR
1– 3 ("a" and "c")	ND		(–)	(–)	ND		(–)	(–)	ND	
4-a1	(–)	(–)	(–)	(–)	(+)	(+)	(–)	(–)	(–)	(–)
4-a2	(–)	(–)	(–)	(–)	(+)	(+)	(+)	(–)	(–)	(–)
4-a3	(–)	(–)	(–)	(–)	(–)	(+)	(–)	(+)	(–)	(–)
4-c	(–)	(–)	(–)	(–)	(+)	(+)	(–)	(+)	(–)	(–)

a1–3 and c: individual animal.
(–): Negative.
(+): Positive.
ND: Not determined.

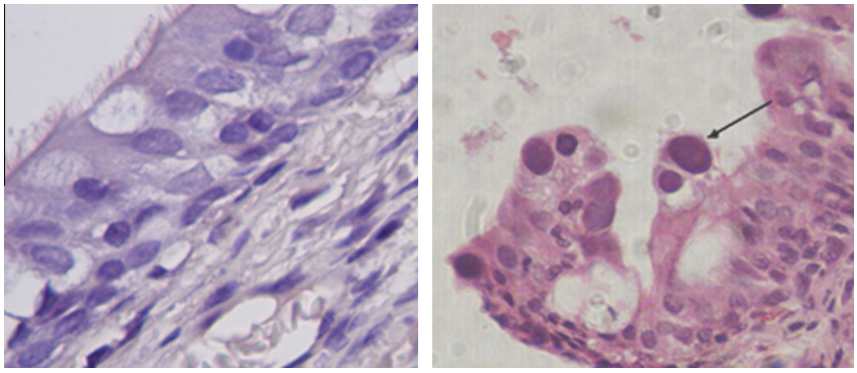

Fig. 3. Nasal turbinate section of one mock-inoculated (left) and virus-inoculated pregnant rabbit of Group 3 (right). Arrows indicate typical inclusion bodies of infection observed in virus-inoculated rabbits (HE,100X).

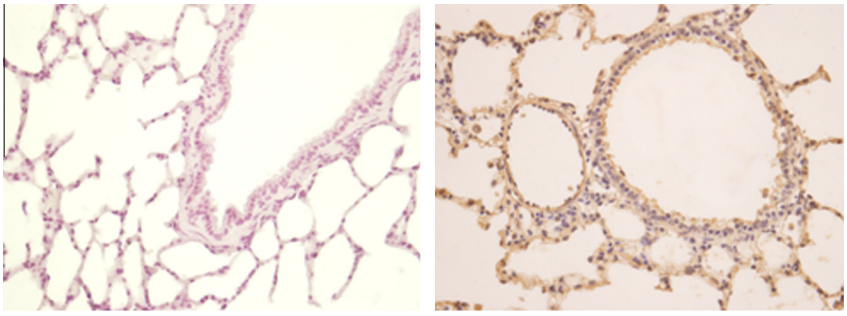

Fig. 4. Immunohistochemistry in lungs. Negative reactivity in the bronchiolar epithelium of a mock-inoculated rabbit (left) and positive reactivity in the bronchiolar epithelium of a virus-inoculated pregnant rabbit of Group 3 (right) (20X).

liver. No histopathological changes were found in the remaining organs studied. Areas of emphysema and pneumonic foci with macrophages, neutrophils and eosinophils were observed in the lungs of one female from Sub-group "a" of Group 3 that died at 4 dpi (6 or 7 days before parturition). Turbinate samples showed typical IB (Fig. 3) and immunohistochemical analysis of the lungs showed positive reactivity in the bronchiolar epithelium, thus confirming the presence of specific viral antigen (Fig. 4). The liver showed a moderate number of macrophages and lymphocytes in the portal spaces, whereas the kidneys showed focal areas of glomerular hyperaemia, cortical and cortico-medullar haemorrhagic foci and mononuclear infiltration, consistent with suppurative interstitial nephritis. The uterus showed some dilated endometrial glands with severe hyperaemia and moderate mononuclear cell infiltration in the lamina propria of the endometrial surface. The liver of four foetuses of one rabbit from Sub-group "a" of Group 3 and the hypokinetic newborn of one rabbit of the same Sub-group showed fatty degenerative changes. The placentas from two rabbits from Sub-group "a" of Group 2 and one of the same Sub-group but of Group 3 showed necrotic, autolytic and hyperaemic areas and mineralization foci in the arteries. However, all samples were immunohistochemically negative. No lesions were found in the placentas and organs of dead foetuses of two rabbits from Sub-group "a" of Group 4. However, the immunohistochemical analysis showed positive reactivity to viral antigen in decidual uninucleated cells and in some structures of the placental labyrinth, particularly around the maternal blood spaces and in the foetal blood vessels (Fig. 5).

4. Discussion and conclusions

Although several BoHV-1.1 studies used the rabbit as an experimental model to reproduce the infection (Lupton et al., 1980; Rock and Reed, 1982; Kelly, 1977), none have analysed the systemic effects after using i.n. inoculation, the natural route of BoHV-1.1 entry in cattle. Our aim was to describe the systemic effects induced by BoHV-1.1 in intranasally inoculated pregnant and non-pregnant rabbits. The rabbit model has several features that closely resemble the infection in the natural host, such as the production of respiratory distress and a viraemic phase to gain access to the maternal-foetal interface. In the present study, BoHV-1.1 infection was clinically confirmed in all the virus-inoculated animals (Subgroups "a" and "c" of each Group). Clinical signs appeared on day 1 of infection, in contrast with the more extended period of incubation (until day 5 pi) observed in the natural host (Muylkens et al., 2007). All virus-inoculated rabbits (at different stages of pregnancy and non-pregnant females) showed respiratory clinical signs. Although it was not possible to record minor changes in body temperature, no increases were recorded during the clinical period of the infection. These data are in accordance with the results obtained by Lupton et al. (1980), who reported that fever lasted for less than 24 h and was not always present in all infected animals.

The humoral response results agreed with those of Honda et al. (1991) and the natural response during infection in cattle. In the present work, the highest peak of antibodies was detected between 30–35 dpi, and antibodies were not detected from 80–90 dpi to the end of the experiment. In addition, antibodies were also detected in suckling rabbits, thus confirming maternal infection and the transmission of passive immunity by the colostrum. Honda et al. (1991) measured the antibody production by ELISA and neutralisation test in intravenously or intratracheally virus-inoculated rabbits, and detected a peak between 3–4 weeks pi and undetectable levels at nine weeks pi. These animals showed no clinical signs during the experimental period.

The clinical signs, the positive detection of humoral immune response, the presence of IB in nasal turbinates and the positive

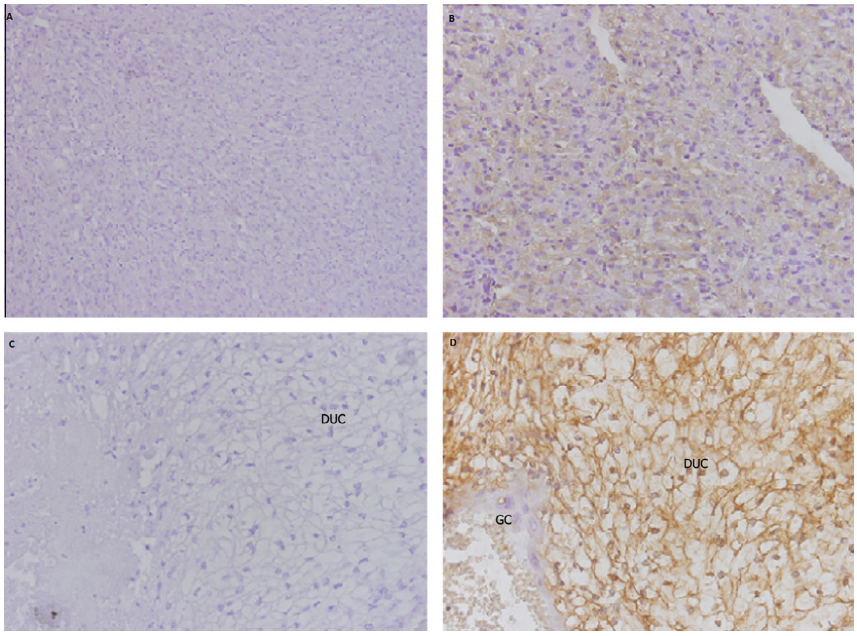

Fig. 5. Immunohistochemistry in placentas. Placentas of virus-inoculated pregnant rabbits of Group 4. (A). Negative reactivity in the placental labyrinth of a non-infected rabbit (40X); (B). Positive reactivity (brown) around maternal blood space and the foetal blood vessels in the placental labyrinth of a virus-inoculated rabbit (40X); (C). Negative reactivity in uninucleated decidual uterine cells (DUC) in the placenta of a mock-inoculated rabbit (40X); (D). Positive staining (brown) of uninucleated decidual uterine cells (DUC) and negative staining of giant cells (GC) in the placenta of a virus-inoculated rabbit (40X). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

immunohistochemical reactivity in the lungs of dead animals strongly support the occurrence of respiratory infection caused by BoHV-1.1. Although in the present work we did not evaluate the cellular immune response, the virus isolation and infectivity titration showed active virus replication and excretion.

In contrast with the results reported by other authors, neurological signs were not observed in the rabbits of any Sub-group or Group. Since the neurological disease was experimentally reproduced in rabbits of 30–45 days old (Spilki et al., 2002), age seems to be a conditioning factor of the neurological clinical form. Our study was carried out using adult rabbits, but no samples of the central nervous system were included in the analysis. However, in a previous study, we reported that adult rabbits inoculated with BoHV-1.1 developed no neurological signs or lesions, and that virus isolation from brain samples was negative. In that study, classical neurological signs and positive virus isolation from brain samples were observed only in weaned rabbits at 6–10 dpi (Valera et al., 2008).

Tikoo et al. (1995) reported the presence of secondary bacterial infection foci in the lungs of naturally infected cattle. In the present study, we observed similar changes in the lungs of infected animals. In some virus-inoculated pregnant rabbits, respiratory clinical signs were followed by a severe pneumonia ending in death. The pathological findings in their lungs included typical lesions compatible with concomitant bacterial infections (Tikoo et al., 1995), and viral antigens were immunohistochemically detected in the bronchial epithelium.

The ability of BoHV-1.1 to initiate a viraemic phase in the natural host has been previously shown (Fuchs et al., 1999). Engels and Ackermann (1996) reported that virus isolation is difficult since few viral particles either bind to or are transported in leukocytes. Tikoo et al. (1995) showed that the viraemia is shorter in cattle and as a result, viral recovery from leukocytes is not always successful. In the present work, we detected virus by virus isolation and PCR from plasma rich in leukocytes of virus-inoculated pregnant and non-pregnant rabbits at ~60 and ~72 h pi.

Abortion caused by BoHV-1.1 in the rabbit experimental model has only been reported as an accidental finding. Up to now, the rabbit has not been completely validated as an animal model to study abortigenic infection. Lupton et al. (1980) reported abortions after the intravenous inoculation of BoHV-1 in some rabbits, but none of the animals showed respiratory signs. The analysis of animals from Sub-group "a" of Group 1 showed respiratory clinical signs and the development of a specific humoral response that was confirmed by ELISA. These rabbits were negative to the pregnancy palpation test and none reached parturition. Low pregnancy rates have been previously reported in infected cattle and have been associated with the endometritis caused by artificial insemination of infected semen (Tikoo et al., 1995). We suggest that, as in the natural host, viral infection may also be responsible for the lack of pregnancy. However, it is important to take into account that the assessment and confirmation of early pregnancy in rabbits is difficult due to the formation of special uterine folds that envelop blastocysts (Amoroso, 1952). Only progressive and daily histological studies of the uteri during these first days of gestation might confirm pregnancy or lack of pregnancy.

The virus-inoculated animals of Groups 2 and 3 showed respiratory clinical signs similar to those observed in rabbits of Group 1. However, three pregnant rabbits (two of Group 2 and one of Group 3) died with signs of pneumonia. Samples from foetuses or newborn offspring from these dead or parturient rabbits were negative for virus isolation and PCR, and IB were found at histopathology. These results are consistent with those observed in cattle, in which aborted foetuses showed few macroscopic lesions, and no histopathological changes, such as IB, to confirm BoHV-1.1 infection (Owen et al., 1964). As in cattle, foetal death occurred within 24–48 h pi and the delayed expulsion of foetuses produced autolytic changes, thus making histopathological analysis of the lesions difficult. A negative PCR reaction has been well-documented in animals experimentally inoculated with BoHV-4. The loss of infectivity of viral particles for sample autolysis has also been reported, explaining negative virus isolation in cell cultures (Naeem et al., 1990, 1991).

Differences between cattle and rabbit placentas must be considered before extrapolating or comparing results. Histologically, the

bovine placenta is epitheliochorial (synepitheliochorial) and foetal-maternal contact is strong due to the formation of placentomes (Wooding and Burton, 2008). Although the rabbit placenta is hemodichorial, foetal-maternal contact is still strong (Björkman et al., 1989). There are few reports regarding placental lesions in BoHV-1-infected animals, and none of the changes described are pathognomonic of the infection. In infected cattle, Gibbs and Rweyemamu (1977) found degenerative changes in the cotyledons of placentas, and extensive perivascular oedema and coagulative necrosis in the chorioallantoic membrane, associated with progressive and severe placental vascular alterations and possible to detachment and subsequent abortion. Smith (1997) found no histopathological lesions in infected placentas, and isolated the virus in the placentas of a few animals but not in their foetuses. This author concluded that the cause of abortion was cessation of placental circulation, which explained the absence of viral lesions in the uterus or placenta. These results are consistent with those described for the placentas of animals of Groups 2 and 3 of the present study, in which necrotic foci and areas of hyperaemia and autolysis were detected. Negative immunohistochemical results have been previously reported in the placentas of infected cattle (Smith et al., 1989). In studies of other herpesviruses, such as the abortigenic strains of *Equid herpesvirus* 1, viral DNA was detected in placentas by PCR, but viral antigen could not be detected by IHC, possibly due to the suppression of viral replication in the placenta by unknown mechanisms (Mukaiya et al., 2000).

Liver samples from dead foetuses and placentas of animals of Group 4 were positive for PCR. This may be explained by the fact that BoHV-1.1 may reach the foetus through the umbilical cord, as has been reported in cattle (Smith, 1997). Besides, positive immunohistochemical reactivity was also found in the placentas of these animals, parturition did not occur in one virus-inoculated rabbit from Group 4, possibly due to the abortigenic effect of BoHV-1.1. However, this assumption could not be confirmed in more animals.

The present rabbit experimental model was appropriate to evaluate the systemic infection induced by i.n inoculation of BoHV-1.1. Virus-inoculated animals showed respiratory disease and virus was isolated from nasal swabs, plasma rich in leukocytes (thus confirming viraemia) and dead foetuses. We detected a positive humoral immune response, and found histopathological lesions in the lungs. Although no histopathological changes were observed in the infected placentas, PCR and IHC results were positive, thus indicating viral dissemination to the placenta by the haematogenous route.

To our knowledge, this is the first report on the systemic infection and abortion induced by i.n inoculation of BoHV-1.1 in rabbits. Taking into account the similarities of the infection between the natural host and rabbits, we suggest that this experimental model might be useful to study different reproductive failures induced by BoHV-1.1 infection.

Acknowledgments

This study was supported by grants from CIC PBA and the Department for Science and Technology at the National University of La Plata (Buenos Aires, Argentina). We thank Prof. Cecilia Carbone (Department of Laboratory Animals) for her professional assistance, and the Faculty of Veterinary Sciences, National University of La Plata (FCV-UNLP) for providing the facilities. We also thank Dr Cristina Seki (Center of Animal Virology – CEVAN – Buenos Aires, Argentina) and Kuniaki Suzuki PhD (PROVETSUR – Japan International Cooperation Agency) for providing us with the monoclonal antibody and the STATA License No. 81990533760 (Stata Corp, 2005., Version 9, College Station, TX, USA), respectively, and Dr Enrique Portiansky (FCV-UNLP, CCT-CONICET La Plata, Argentina) for helping in the image processing.

References

Alegre, M., Nanni, M., Fondevila, N., 2001. Development of multiplex polymerase chain reaction for the differentiation of bovine Herpesvirus-1 and 5. Journal of Veterinary Medicine B 48, 613–621.
Amoroso, E.C., 1952. Placentation. In: Parkes, A.S. (Ed.), Marshall's Physiology of Reproduction, second ed. Longmans Green, London, pp. 127–311.
Awan, A.R., Baxi, M., Field, H.J., 1995. EHV-1 induced abortion in mice and its relationship to stage of gestation. Research in Veterinary Science 59, 139–145.
Babic, N., Mettenleiter, T.C., Ugolini, G., Flammand, A., Coulon, P., 1994. Propagation of pseudorabies in the nervous system of the mouse after intranasal inoculation. Virology 204, 616–625.
Björkman, N., Dantzer, V., Leiser, R., 1989. Comparative placentation in laboratory animals. A review. Scandinavian Journal of Laboratory Animal Science 16, 129–158.
Brown, G.A., Field, H.J., 1990. A reliable method for establishing viral infection in the rabbit by intranasal inoculation. Journal of Virological Methods 27, 341–346.
Chowdhury, S.I., Lee, B.J., Mosier, D., Sur, J.H., Osorio, F.A., Kennedt, G., Weiss, M.L., 1997. Neuropathology of Bovine Herpesvirus Type 5 (BHV-5) meningoencephalitis in rabbit seizure model. Journal of Comparative Pathology 117, 295–310.
Engels, M., Ackermann, M., 1996. Pathogenesis of ruminant herpesvirus infections. Veterinary Microbiology 53, 3–15.
Fuchs, M., Hubert, P., Detterer, J., Rziha, H.J., 1999. Detection of bovine herpesvirus type 1 in blood from naturally infected cattle by using a sensitive PCR that discriminates between wild-type virus and virus lacking glycoprotein E. Journal of Clinical Microbiology 37, 2498–2507.
Gibbs, E.P.J., Rweyemamu, M.M., 1977. Bovine herpesviruses. The Veterinarian Bulletin 47, 317–332.
Honda, E., Taniguchi, T., Watanabe, M., Kumagai, T., 1991. Detection of bovine rhinotracheitis virus antibody by neutralizing test and ELISA in experimentally infected rabbits. Journal of Veterinary Medicine B 38, 55–59.
Kelly, D.F., 1977. Experimental infection of rabbits with the virus of infectious bovine rhinotracheitis. The British Journal of Experimental Pathology 58, 168–176.
Lee, B.J., Weiss, M.L., Mosier, D., Chowdhury, S.I., 1999. Spread of bovine herpesvirus type 5 (BHV-5) in the rabbit brain after intranasal inoculation. Journal of Neurovirology 5, 474–484.
Lupton, H.W., Barnes, H.J., Reed, D.E., 1980. Evaluation of the rabbit as a laboratory model for infectious bovine rhinotracheitis virus infection. The Cornell Veterinarian 70, 77–95.
Meyer, G., Lemaire, M., Lyaku, J., Pastoret, P.P., Thiry, E., 1996. Establishment of a rabbit model for bovine herpesvirus type 5 neurological acute infection. Veterinary Microbiology 51, 27–40.
Mukaiya, R., Kimura, T., Ochiai, R., Eada, R., Unemura, T., 2000. Demonstration of equine herpesvirus-1 gene expression in the placental trophoblasts of naturally aborted equine fetuses. Journal of Comparative Pathology 123, 119–125.
Muylkens, B., Thiry, J., Kirten, P., Schynts, F., Thiry, E., 2007. Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis. Veterinary Research 38, 181–209.
Naeem, K., Caywood, D.D., Werdin, R.E., Goyal, S.M., 1990. Evaluation of pregnant rabbits as a laboratory model for bovid herpesvirus-4 infection. American Journal of Veterinary Research 51, 640–644.
Naeem, K., Murtaugh, M.P., Goyal, S.M., 1991. Tissue distribution of bovid herpesvirus-4 in inoculated rabbits and its detection by DNA hybridization and polymerase chain reaction. Archives of Virology 119, 239–255.
Osorio, F.A., Reed, D.E., Rock, D.L., 1982. Experimental infection of rabbits with bovine herpesvirus-4: acute and persistent infection. Veterinary Microbiology 7, 503–513.
Owen, N.V., Chow, T.L., Molello, J.A., 1964. Bovine fetal lesions experimentally produced by infectious bovine rhinotracheitis virus. American Journal of Veterinary Research 25, 1617–1626.
Reed, L.J., Muench, H., 1938. A simple method of estimating fifty per cent end points. The American Journal of Hygiene 27, 493–497.
Rock, D.L., Reed, D.E., 1982. Persistent infection with bovine herpesvirus type 1: rabbit model. Infection and Immunity 35, 372–373.
Rock, D.L., Beam, S.L., Mayfield, J.E., 1987. Mapping bovine herpesvirus type 1 latency-related RNA in trigeminal ganglia of latently infected rabbits. Journal of Virology 61, 3827–3831.
Silva, A.M., Flores, E.F., Weiblen, R., Canto, M.C., Irigoyen, L.F., Roehe, P.M., Souza, R., 1999. Revista de Microbiologia 30, 22–31.
Smith, P.C., 1978. Experimental infectious bovine rhinotracheitis virus infections of English ferrets (Mustela putorius furo L). American Journal of Veterinary Research 39, 1369–1372.
Smith, G.H., Collins, J.K., Carman, J., 1989. Use of an immunoperoxidase test for the detection of bovine herpesvirus-1 in aborted fetal tissue. Journal of Veterinary Diagnostic Investigation 1, 39–44.
Smith, K.C., 1997. Herpesviral abortion in domestics animals. Review Veterinary Journal 153, 253–268.
Spilki, F.R., Esteves, P.A., Franco, A.C., Lima, M., Holz, C.L., Batista, H.B.R., Driemeier, D., Flores, E.F., Weiblen, R., Roehe, P.M., 2002. Neurovirulencia e

neuroinvasividade de herpesvirus bovinos tipo 1 e 5 em coelhos. Pesquiza Veterinaria Brazileira 22, 58–63.
Tikoo, S., Campos, M., Babiuk, L., 1995. Bovine herpesvirus 1 (BHV-1): biology, pathogenesis and control. Advances in Virus Research 45, 191–223.
Valera, A.R., Galosi, C.M., Echeverría, M.G., Silvestrini, M.P., Romero, J.R., Nosetto, E.O., 1999. Desarrollo y Estandarización de un ELISA indirecto para la detección de anticuerpos contra el herpesvirus bovino tipo 1. Avances en Medicina Veterinaria 13, 21–25.
Valera, A.R., Pidone, C.L., Massone, A.R., Quiroga, M.A., Riganti, J.G., Corva, S.G., Galosi, C.M., 2008. A simple method of infecting rabbits with *Bovine herpesvirus 1* and *5*. Journal of Virological Methods 150, 77–79.
Wooding, P., Burton, G., 2008. Eutheria: synepitheliochorial placentation Ruminants (Ewe and cow). In: Wooding, P., Burton, G. (Eds.), Comparative Placentation. Structure, Function and Evolution. Springer-Verlag, Berlin Heidelberg, pp. 133–168, Chapter 6.