Reproduction in Domestic Animals

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Journal:	*Reproduction in Domestic Animals*
Manuscript ID	Draft
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
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Subject Area:	Goat, Abortion, Histopathology, Real-time PCR, Spain, Caprine alphaherpesvirus 1

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Short Running Title

First report of a CpHV-1 abortion outbreak in goats in Spain

Contents:

An abortion outbreak occurred in a Murciano-Granadina goat flock in Almeria Region in Spain where 80 pregnant females aborted. All bacteriological and parasitological examinations resulted negative whereas virological investigations showed the presence of Caprine alphaherpesvirus 1 DNA in the pathological specimens from aborted fetuses. Sequence analysis revealed that the DNA was highly close related to the Swiss strain E-CH (99,7%) and a little less extent to the Italian BA.1 strain (99,4%). Histopathologic examination revealed multifocal, well circumscribed, 50-200 µm diameter foci of coagulative necrosis in the liver, lungs and kidneys of 3 fetuses. In the periphery of the necrosis there were frequently epithelial cells with the chromatin emarginated by large, round, amphophilic intranuclear viral inclusion bodies. The source of the infection in the flock could not clearly find out even some hypothesis were formulated. This seems to be the first report of an abortion storm in a goat flock in Spain.

Key words:

Caprine alphaherpesvirus 1; goat; abortion; histopathology; Real-time PCR; Spain

Introduction

Caprine alphaherpesvirus 1 (CpHV-1), a ruminant alphaherpesvirus closely related to Bovine alphaherpesvirus 1 (BoHV-1), was first isolated from goats in 1974 and further characterized in 1975 (Berrios et al., 1975; Saito et al., 1974). The pathogenesis of the infection is not yet completely understood, although the virus is believed to infect goats via the nasal or reproductive via, having high tropism for the genital tract (Tempesta et al., 2001; Tempesta et

al., 2004). Viral infection can induce vulvovaginitis, balanophosthitis, respiratory disease and occasionally abortions in adult goats (Grewal et al., 1986; Horner et al., 1982; Tarigan et al., 1987; Williams et al., 1997), whereas enteritis and systemic infection occur more frequently in kids (Roperto et al., 2000; Waldvogel et al., 1981). Similar to other alphaherpesvirus, CpHV-1 can induce subclinical disease and be latent for long periods of time. However, reactivation of the virus under natural conditions has been shown to be rare and only occurring in animals as a consequence of stressing factors such as estrus in the presence of low neutralizing antibody titres (Tempesta et al., 1998). Reactivation of infection has been induced experimentally in latently infected goats after treatment with high doses of dexametasone (Buonavoglia et al., 1996).

Although CpHV-1 infections have a worldwide distribution, outbreaks of clinical disease have rarely been observed, mainly consisting of systemic disease in kids and genital lesions in adult animals (Belton, 1992; Grewal et al., 1986; Horner et al., 1982; Piper et al., 2008; Roperto et al., 2000; Saito et al., 1974). CpHV-1 as a determinant of abortion in goats accounts for only a low percentage of the total causes (McCoy et al., 2007) and it has only been described sporadically (Chénier et al., 2004; McCoy et al., 2007; Uzal et al., 2004; Williams et al., 1997).

CpHV-1 was isolated in the south of Spain (Cordoba) in two seropositive goats without clinical signs and the identification of the virus was achieved after reactivation with corticosteroids (Keuser et al., 2004). In this report, we describe for the first time in Spain an outbreak of abortion in goats from which CpHV-1 was successfully identified as the causative agent by histopathology and PCR.

Materials and Methods

All the investigations and procedures were made following international guidelines and ethics.

Case report

The abortion outbreak occurred in a goat herd of Murciano-Granadina breed in Almeria (South-East of Spain). The herd consisted of a total of 1050 goats kept in an intensive dairy productive system without any contact with other species. The herd had no previous history of abortion over the past few years, there were no recent factors of stress (feeding changes, management, etc.) and introduction of new animals had not occurred over the last five years. During the last two years, female replacement goats were vaccinated with an attenuated live vaccine against *Chlamydophila abortus*. A total of 80 abortions occurred over a period of 45 days during the autumn birth season (October-November). The abortion occurred at different gestation periods. There were no other clinical signs, including genital lesions, in any of the adult goats. Some of the kids were stillbirth or born weak. In the case of twins, usually one of the kids survived and the other died at birth. In three aborted fetuses the necropsy was carried out and the respective placentas were also observed for gross pathological changes. Samples of the brain, lungs, liver, kidney, placenta and sera from does and fetuses were appropriately collected for investigations.

Laboratory investigations

Samples from placentas and organs were fixed in 10% non-buffered formalin, routinely embedded in paraffin and processed for histopathology. Slides were stained with haematoxilin-eosin, Gram and Stamp.

In order to detect CpHV-1 DNA, paraffin blocks from samples previously showing necrotic foci were used. DNA samples were extracted from tissue sections embedded in paraffin using a DNA extraction Kit.

The DNA extracts were tested by a CpHV-1 real-time PCR assay, following the method developed by Elia et al., 2008. Briefly, real-time PCR was carried out in a 25-µl reaction volume containing 12.5 µl of PCR buffer 600 nM of primers CpHV-For and CpHV-Rev, 200 nM of probe CpHV-Pb (Table 1) and 10 µl of DNA. Serial ten-fold dilutions (from 10^9 to 10^2 DNA copies/10 µl of standard DNA) of a plasmid (containing the nearly full-length gC gene of CpHV-1 were used to generate a standard curve. Duplicates of the CpHV-1 standard dilutions and DNA templates were simultaneously subjected to real-time analysis. An exogenous internal control, consisting of 10,000 copies of canine parvovirus type 2 (CPV-2) DNA per ml of lysis buffer, was added to each sample prior to DNA preparation to control for PCR inhibition. Real-time PCR was carried out in a commercial PCR equipment. The following thermal cycle protocol was used: activation of Taq DNA polymerase at 95°C for 10 min and 45 cycles consisting of denaturation at 95°C for 1 min, primer annealing and extension at 70°C for 1 min.

In order to confirm the real-time PCR results and characterize at the molecular level the detected CpHV-1 strain, a PCR was performed as described elsewhere (Tempesta et al., 1999). Briefly, a pair of primers corresponding to the sequences 632-653 and 1046-1027 of the gC gene of CpHV-1 strain BA.1 (GenBank accession number AY821804) was chosen. PCR was carried out in a total volume of 25 µl containing 5 µl of DNA sample, 2.5µl of PCR buffer 10x, 1.5 mM MgCl2, 1.25 mM of each oligonucleotide triphosphate, 200 µM of each primer, 1.5 U of a commercial Taq 2.5 µl of glycerol and sterile water up to 25 µl. The thermal profile consisted of a 1 min at 94°C, 40 cycles at 94°C for 1 min (denaturation), 70°C for 1 min (annealing) and 72°C for 1 min (polymerization) followed by a final extension at 72°C for 10

min. Ten microliters of the PCR products were analyzed by electrophoresis in 1.5% agarose gel and visualized by UV light after ethidium bromide staining.

The PCR products were purified in columns and sequenced using a Dye Terminator Cycle Sequencing Kit with a DNA sequencer equipment. The obtained nucleotide and amino acid sequences were analyzed using the analytical tools by the National Center for Biotechnology Information (NCBI) and align by the program ClustalW and FASTA.

Results

Post-mortem findings and histopathology

There were no macroscopic lesions in any of the fetuses or the placentas and no evidence of a common causative agent of abortion in goats was found. Examination under the microscope revealed multifocal, well circumscribed, 50-200 μm diameter foci of coagulative necrosis in the liver, lungs and kidneys of the 3 fetuses (Fig. 1A). Foci of necrosis were characterized by a center of necrotic tissue and degenerating neutrophils and abundant karyorrhectic and karyolitic debris rimmed by a mild inflammatory cell infiltrate mainly compose of lymphocytes and macrophages. In the periphery of the necrosis there were frequently epithelial cells with the chromatin emarginated by large, round, amphophilic intranuclear viral inclusion bodies (Fig. 1B). In the brain, similar smaller lesions were sporadically observed, but no intranuclear viral inclusion bodies were observed in this location.

Molecular detection and characterization of CpHV-1

Real-time PCR showed the presence of CpHV-1 DNA in the pools obtained from liver, lung and kidney of both fetuses, with viral DNA titers of 3.39 and 5.16×10^{-7} DNA copies/ml^{-1} of template, respectively. Gel-based PCR confirmed the results obtained by real-time PCR and

allowed to obtain a 390-nucleotide gC fragment for subsequent sequence analysis. This showed that the two Spanish CpHV-1 strains detected in aborted fetuses were identical (100% of nucleotide identity). The closest genetic relatedness was found with the Swiss prototype strain E-CH (99.7% of nucleotide identity), whereas the Spanish strains were slightly less related to the Italian isolate Ba-1 (99.4% of nucleotide identity).

Testing for other pathogens

ELISA test was performed on sera from does and fetuses to test for *Toxoplasma gondii* antibodies, giving negative results. Gram and Stamp staining on direct impression smears from fetal tissues did not detect any specific bacteria. Standard bacteriological cultures were performed with tissues from the fetus; they all failed to grow any organism. No Campylobacter spp., Coxiella spp., Chlamydia spp., Toxoplasma were detected. On the basis of the histopathological lesions and RT-PCR results, a diagnosis of abortion caused by Caprine alphaherpesvirus type 1 (CpHV-1) was confirmed.

Discussion

Infectious causes of abortion in goats are widespread and predominantly of bacterial origin (Moeller, 2001). Differential diagnoses based on the gross lesions observed in the placenta and the fetus could be tentatively done. In fact, the histopathological areas of necrosis observed in several tissues, with limited inflammatory response around the necrotic foci and the presence of characteristic intranuclear viral inclusion bodies pointed directly towards a herpesvirus infection. Other common causative agents of abortion in goats were all ruled out after negative bacteriological culture, ELISA and absence of characteristic pathological changes of these etiological agents. Infection with CpHV-1 in goats can yield a limited variety of clinical signs in the herd. Outbreaks of vulvovaginitis usually occur without being associated with signs in

the fetus or abortion (Belton, 1992; Grewal et al., 1986; Horner et al., 1982). On the other hand, outbreaks of abortion usually occur without genital pathologic changes (vulvovaginitis or balanoposthitis) in adults goats, (McCoy et al., 2007; Tempesta et al., 2004; Williams et al., 1997) as reported in the present note.

The source of infection in this herd remains uncertain; the farmer had not introduced new animals in the herd over the previous years. As observed in natural and experimental infections and similar to other herpesvirus infections, CpHV-1 could remain latent in some animals for long time periods (Tempesta et al., 1999). Even though no special treatments or stressful situations were reported in the flock involved in the described outbreak, reactivation of a latent infection could not be ruled out (Buonavoglia et al., 1996; Tempesta et al., 1998). CpHV-1 had been previously isolated from two seropositive goats in the south of Spain, however, there were no clinical signs of genital pathology or abortion reported in either of the animals from which the virus was isolated (Keuser et al., 2004). The presence of CpHV-1 infection in two goat flocks in the south of Spain that were geographically separated (350 km far from each other), along with the occurrence of overt disease in one case, suggests a high CpHV-1 prevalence in that region having a large goat population, as it occurs in other Mediterranean countries (Keuser et al., 2004; Koptopoulus et al., 1988; Thiry et al., 2008). Although CpHV-1 has been previously identified in Spain, to our knowledge this is the first description of abortions caused by this agent in the Iberian Peninsula. In our opinion, this infection should be included in the diagnostic panels for common causes of abortion in goats in Spain and serological studies would be necessary to know the widespread of the infection, especially in a region with a high goat population.

Conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions

All Authors listed contributed at the search.

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Figure 1: **A**: Several foci of coagulative necrosis in liver parenchyma, up to 500 µm in diameter with a mild inflammatory cell infiltrate at the periphery. H&E. 4x; **B**: Detail of the periphery of a necrotic focus showing karyorrhetic, karyolitic, nuclear pyknosis and parietal hyperchromatosis in hepatocytes. H&E. 40x; **C**: Intranuclear inclusion body (arrow) in a hepatocyte with marginated nuclear chromatin. H&E. 50x; **D**: Lung. Similar focus of coagulative necrosis to those observed in the liver. H&E. 10x.

Table 1. Oligonucleotides used in the CpHV-1 Real-Time PCR and gel-based PCR assays

Primer/probe	Sense	Position [a]	Amplicon size
CapIII[b]	+	632-653	414
CapIV[b]	-	1027-1046	
CpHV-For[c]	+	897-918	
CpHV-Rev[c]	-	959-979	82 bp
CpHV-Pb[c]	+	926-951	

[a] Oligonucleotide position is referred to the sequence of CpHV-1 reference strain Ba.1 (accession: AY821804)
[b] Gel-based PCR
[c] Real-Time PCR

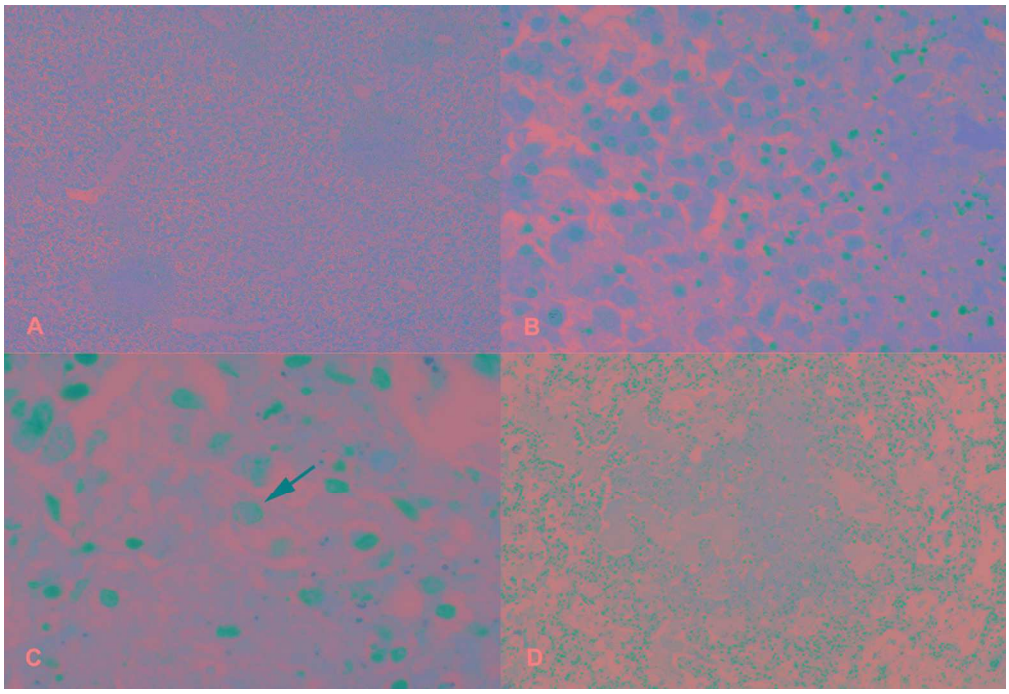

Figure 1: A: Several foci of coagulative necrosis in liver parenchyma, up to 500 µm in diameter with a mild inflammatory cell infiltrate at the periphery. H&E. 4x; B: Detail of the periphery of a necrotic focus showing karyorrhetic, karyolitic, nuclear pyknosis and parietal hyperchromatosis in hepatocytes. H&E. 40x; C: Intranuclear inclusion body (arrow) in a hepatocyte with marginated nuclear chromatin. H&E. 50x; D: Lung. Similar focus of coagulative necrosis to those observed in the liver. H&E. 10x.

179x122mm (300 x 300 DPI)