



# Detección de anticuerpos contra lentivirus de pequeños rumiantes en fetos ovinos y caprinos

## Detection of antibodies against small ruminant lentiviruses in ovine and caprine fetuses

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### Abstract

The presence of antibodies against Maedi-Visna (MV) and caprine arthritis encephalitis (CAE) was evaluated in ovine and caprine fetuses obtained from pregnant females slaughtered in abattoirs. Sera from 54 caprine fetuses and 65 ovine fetuses were collected and evaluated by the indirect ELISA technique. Antibodies were detected in five caprine fetuses of 80 days of gestation and in four ovine fetuses of 90 to 100 days of gestation; three caprine fetuses and two ovine fetuses of 60 to 65 days of gestation were suspicious. The possibility of uterine infection with these retroviruses is confirmed, and its consequences in current disease control and eradication strategies are discussed.

**Key words:** RETROVIRUS, SHEEP, GOAT, SMALL RUMINANTS, CAPRINE ARTHRITIS ENCEPHALITIS, MAEDI-VISNA, FETAL IMMUNITY, TRANSPLACENTAL INFECTION, UTERINE INFECTION.

### Resumen

Se evaluó la presencia de anticuerpos contra los virus del Maedi-Visna (MV) y la artritis encefalitis caprina (AEC) en fetos ovinos y caprinos obtenidos de hembras gestantes sacrificadas para consumo en México. Se recolectaron sueros de 54 fetos caprinos y 65 fetos ovinos que se evaluaron mediante ELISA indirecto. Se detectaron anticuerpos en cinco fetos caprinos de 80 días de gestación y en cuatro fetos ovinos de 90 a 100 días; tres fetos caprinos y dos ovinos de 60 a 65 días de gestación resultaron sospechosos. Se confirma la posibilidad de la infección uterina por estos retrovirus y se discuten sus consecuencias en las actuales estrategias de control y erradicación de las enfermedades.

**Palabras clave:** RETROVIRUS, OVINOS, CAPRINOS, PEQUEÑOS RUMIANTES, ARTRITIS ENCEPHALITIS CAPRINA, MAEDI-VISNA, INMUNIDAD FETAL, INFECCIÓN TRANSPLACENTARIA, INFECCIÓN UTERINA.

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## Introduction

Small ruminant lentiviruses (SRLV) cause chronic degenerative inflammatory lesions in various organs, such as joints, lungs, brain, and the mammary gland of sheep and goats. The two most relevant and widely studied diseases caused by SRLV are caprine arthritis encephalitis (CAE) and Maedi-Visna (MV), which were considered as different species-specific diseases, but recent work on genomic relationships of these viruses and its follow up in mixed flocks of sheep and goats, indicates that transmission of these viruses between species is possible.<sup>1-4</sup> The clinical picture is progressive, and it usually requires several months and even years to develop. Seroconversion also occurs tardily, it may appear in a few weeks or up to two years post-infection. The main target cells are monocytes/macrophages and bone marrow is considered as the virus reservoir organ. Shortly after infection, the virus enters into a period of latency or restricted replication and the amount of viral particles in blood and secretions tends to be extremely low.<sup>5,6</sup>

Colostrum ingestion is considered as the main route of transmission for CAE and as a way of MV transmission; the virus is vehiculated in the macrophages present in this secretion and in the milk of infected mothers.<sup>7</sup> Regarding MV, transmission by respiratory route through aerosols contaminated with infected cells from ewes to their lambs is recently hierarchized.<sup>8,9</sup>

The possibility of venereal and intrauterine transmission has gained importance due to the increasingly widespread use of assisted reproduction, artificial insemination and embryo transfer techniques in small ruminants, which involve additional risks of infection in regions and flocks free from these diseases.<sup>6,8,10</sup>

Experimentally, positive results have been accomplished by infecting ovine fetuses of 100 days of gestation with MV virus, achieving challenge virus recovery.<sup>11</sup> It has been observed that caprine offspring obtained by cesarean section and fed with virus-free colostrum may experience seroconversion a few months after birth.<sup>12</sup> The presence of the viral genome and anti-MV serologic response has been demonstrated by PCR and ELISA in lambs without colostrum intake, born to females from naturally infected flocks, and a relationship to antigenemia was observed in sheep with positive lambs. The percentage of positive lambs in these cases does not seem to be insignificant, ranging between 5 and 11%.<sup>13,14</sup> Besides, susceptibility of uterine epithelial cells to SRLV infection has been demonstrated *in vitro*.<sup>15-17</sup> These findings indicate that transplacental infection occurs, but its importance in the prevalence of the disease is uncertain.<sup>6</sup>

The presence of fetal antibodies against SRLV has not been studied until now. Fetal capacity to respond

## Introducción

Los retro-lentivirus de los pequeños rumiantes (LvPR) causan lesiones inflamatorias crónico-degenerativas en diversos órganos: articulaciones, pulmón, cerebro y glándula mamaria de ovinos y caprinos. Las dos enfermedades más relevantes y estudiadas, ocasionadas por LvPR, son la artritis encefalitis caprina (AEC) y el Maedi-Visna (MV); se consideran enfermedades diferentes y específicas de especie, pero los trabajos de evaluación de las relaciones genómicas de estos virus y de seguimiento en rebaños mixtos de ovinos y caprinos, sugieren que es posible la transmisión de estos virus entre especies.<sup>1-4</sup> El cuadro clínico es progresivo y usualmente requiere de meses a años para desarrollarse. La seroconversión también es tardía, puede ocurrir en algunas semanas o hasta los dos años pos-infección. El objetivo principal de estos virus son las células de los monocitos/macrófagos y la médula ósea se considera su órgano reservorio. Poco tiempo después de la infección, el virus entra en un periodo de latencia o de replicación restringida y la cantidad de partículas virales en sangre y secreciones tiende a ser sumamente baja.<sup>5,6</sup>

Se considera que la ingestión del calostro es la principal vía de transmisión de la AEC y una forma de transmisión de MV; los macrófagos presentes en esta secreción y en la leche de las madres infectadas transportan el virus.<sup>7</sup> En el caso de MV, se jerarquiza la transmisión por vía respiratoria a través de aerosoles contaminados por células infectadas, emitidos por las madres y los corderos ya infectados.<sup>8,9</sup>

La posibilidad de la transmisión venérea e intrauterina ha cobrado importancia por el uso cada vez más extendido de las técnicas de reproducción asistida, inseminación artificial y transferencia de embriones en los pequeños rumiantes, las cuales implican riesgos adicionales de infección de regiones y rebaños libres de estas enfermedades.<sup>6,8,10</sup>

Experimentalmente, se han logrado resultados positivos al infectar fetos ovinos de 100 días de gestación con virus de MV, para lograr recuperar el virus de desafío.<sup>11</sup> Se ha observado que crías caprinas obtenidas mediante cesárea y alimentadas con calostro libre de virus, pueden llegar a seroconvertir a los pocos meses del nacimiento.<sup>12</sup> Mediante PCR y ELISA, se ha demostrado la presencia de genoma viral y de respuesta serológica anti MV, en corderos no calostrados, nacidos de hembras provenientes de rebaños naturalmente infectados y se ha observado relación entre la antigenemia de las ovejas y sus corderos positivos. El porcentaje de corderos positivos en estas condiciones no es despreciable, pues varía entre 5 y 11%.<sup>13,14</sup> Por otra parte, se ha demostrado *in vitro*, la susceptibilidad a la infección con LvPR en las células epiteliales del útero.<sup>15-17</sup> Estos

to antigens develops very fast following appearance of the lymphoid organs, but not all antigens have the same capacity to stimulate fetal lymphoid tissue. Lymphocytes are identified in the peripheral blood of bovine fetuses by day 45, IgM+ B cells are identified by day 59, and IgG-producing cells by day 135.<sup>18</sup> Bovine fetuses respond to rotaviruses at 73 days, to parvovirus at day 93, and to the parainfluenza virus at 120 days.<sup>19</sup> Ovine fetuses may produce antibodies against phage FX174 on day 41 of gestation<sup>20</sup> and against Akabane virus since 50 days.<sup>21</sup> In ovine fetuses, it is possible to induce formation of antibodies against SV40 virus on day 90, against phage T4 on day 105, against bluetongue virus on day 122, and against lymphocytic choriomeningitis virus on day 140. The ruminant placenta does not allow passage of maternal antibodies to the fetus; therefore, the presence of a serologic response implies fetal infection.<sup>20</sup>

In Mexico, CAE is widely distributed in commercial dairy flocks, while it does not occur or its prevalence is very low in rural flocks that graze in semi-arid or arid ecosystems,<sup>22-25</sup> possibly because under these extreme conditions the disease is self-limited. Mexico declares itself free from MV, although characteristic pathological pictures have been noted in slaughterhouse materials<sup>26</sup> and an 8% seroprevalence has been demonstrated in native livestock from rural flocks.<sup>27</sup> This situation is a serious inconvenience in ovine exportation for reproduction, since it determined the slaughter of more than 300 animals positive to the virus, which were exported to Colombia in 2007.<sup>28</sup>

The aim of the present work was to evaluate the possible presence of antibodies against SRLV in ovine and caprine fetuses, trying to determine the stage of gestation in which this response can be established.

## Material and methods

Uteri from pregnant females slaughtered in municipal abattoirs in central Mexico were collected and taken to the laboratory for opening under sterile conditions. One hundred nineteen fetuses were obtained, 54 caprine and 65 ovine fetuses. Products were sexed, which resulted in 27 caprine males and 27 caprine females, 43 ovine males and 22 ovine females. The approximate gestation time was determined by measuring the distance from the nape of the neck to the base of tail.<sup>29</sup>

Blood, thoracic fluid, or abdominal fluid, as appropriate, were obtained with a sterile syringe and centrifuged at 1800 *g* for 10 minutes, in order to separate the supernatant (fetal serum) from the cell pack or any tissular detritus. The supernatants were kept under freezing at -70°C until processing.

hallazgos soportan la infección transplacentaria, pero su importancia en la prevalencia de la enfermedad es incierta.<sup>6</sup>

La presencia de anticuerpos fetales contra LvPR no ha sido estudiada hasta ahora. La capacidad fetal para responder a los antígenos se desarrolla muy rápidamente luego de que aparecen los órganos linfoides, pero no todos los antígenos tienen la misma capacidad para estimular el tejido linfoide fetal. Los linfocitos se identifican en la sangre periférica en los fetos bovinos hacia el día 45, las células B IgM+ el día 59, y las productoras de IgG el día 135.<sup>18</sup> Los fetos bovinos responden a los rotavirus a los 73 días; al parvovirus, al día 93 y al virus de parainfluenza 3, a los 120 días.<sup>19</sup> Los fetos ovinos pueden producir anticuerpos contra el fago FX174 el día 41 de gestación<sup>20</sup> y contra el virus de Akabane desde los 50 días.<sup>21</sup> En fetos ovinos es posible inducir la formación de anticuerpos contra el virus SV40 el día 90; contra el fago T4, el 105; contra el virus de lengua azul, el 122, y contra el virus de la coriomeningitis linfocítica, el día 140. La placenta de los rumiantes no permite el paso de anticuerpos maternos a los fetos, por lo que la respuesta serológica positiva implica infección fetal.<sup>20</sup>

En México, la AEC se encuentra ampliamente distribuida en los rebaños lecheros estabulados, mientras que no ocurre, o su prevalencia es muy baja, en los rebaños campesinos, que pastorean en ecosistemas semiáridos o áridos,<sup>22-25</sup> posiblemente porque estas condiciones extremas limitan la enfermedad. México se declara libre de MV, aunque se han señalado cuadros patológicos característicos en muestras de pulmón de matadero<sup>26</sup> y se ha demostrado una seroprevalencia de 8% en ganado nativo de rebaños campesinos.<sup>27</sup> Esta situación se ha convertido en un serio inconveniente en la exportación de ovinos para reproducción y determinó el sacrificio de más de 300 animales positivos al virus, que fueron exportados a Colombia en 2007.<sup>28</sup>

El objetivo del presente trabajo fue evaluar la posible presencia de anticuerpos contra LvPR en fetos ovinos y caprinos, intentando determinar en que etapa de la gestación puede establecerse esta respuesta.

## Material y métodos

En el centro de México se recolectaron úteros de hembras gestantes sacrificadas en mataderos municipales, que se trasladaron al laboratorio para su estudio en condiciones de esterilidad. Se obtuvieron 119 fetos, 54 caprinos y 65 ovinos. Se determinó el sexo de los productos: 27 machos y 27 hembras caprinas, 43 machos y 22 hembras ovinas. Asimismo, se calculó el tiempo aproximado de gestación, midiendo la distancia de la nuca a la base de la cola.<sup>29</sup>

## **ELISA**

The sera obtained were evaluated by the Maedi-Visna/CAEV Serodiagnosis kit, ELISA test serum.\* The kit is used in an indirect ELISA technique, aimed at demonstrating antibodies, mainly of the IgG class, against two viral proteins, the transmembrane proteins (P45, P135, ENV gene) and the recombinant P28 protein, a component of the viral capsid (GAG gene). The use of these highly preserved proteins allows the detection of a wide spectrum of serologic variants of these viruses. Microplate optical density (OD) reading was performed at 450 nm. OD readings were corrected by subtracting the value of reading of the well without antigen from the value obtained on the positive control with antigen. The OD value of each sample was established in percentage terms against the corrected value of the positive control (S/P%). Following the kit manufacturer's directions, samples with an S/P% value equal to, or less than, 40% were considered negative, samples with S/P% between 40% and 50% were considered as suspicious samples, and those with S/P% values equal to, or less than, 50% were considered positive.

## **Counterimmunoelectrophoresis**

The presence of immunoglobulins in fetal serum samples was attempted to be proved by counterimmunoelectrophoresis (CIE) tests. Hyperimmune serum against caprine immunoglobulins in rabbit was prepared. On a 1% agarose gel with barbiturate buffer pH 8.2, a groove was cut in the middle of the gel, with two lateral perforations. Fifty microliters of fetal serum were placed in the lateral perforations. The gel was installed in an electrophoresis chamber and was run at 70 volts for 45 minutes. Three hundred microliters of hyperimmune rabbit serum were subsequently added to the central groove and it was incubated at room temperature for 48 to 72 hours in a moist chamber.

## **Results**

The ages of the fetuses obtained were 50 to 145 days of gestation.

Nine fetuses seropositive to SRLV (7.5%) and five fetuses with suspect SRLV (4.2%) were detected, five were caprine fetuses (9.2%) of approximately 80 days, and four were ovine fetuses (6.1%) of 90 to 100 days of gestation. The suspicious fetuses, three caprine and two ovine fetuses, had a smaller age of approximately 60 days of gestation (Table 1).

On CIE, six of the positive sera formed a precipitation band characteristic of IgM, which did not occur in

Según el caso, se obtuvo sangre y líquido torácico o abdominal con jeringa estéril. Las muestras se centrifugaron a 1800 g por 10 minutos, para separar el sobrenadante (suero fetal) del paquete celular y los posibles detritus tisulares. Los sobrenadantes se conservaron en congelación a -70°C hasta su procesamiento.

## **ELISA**

Los sueros obtenidos fueron evaluados mediante el paquete Maedi-Visna/CAEV Serodiagnosis, ELISA test serum.\* El paquete se empleó con ELISA indirecta, dirigida a demostrar anticuerpos, principalmente de la clase IgG contra dos proteínas virales, la transmembranal (TM, gen ENV) y la proteína P28 recombinante, componente de la cápside viral (gen GAG). El uso de estas proteínas altamente conservadas, permite la detección de un amplio espectro de variantes serológicas de estos virus. La lectura de densidad óptica (DO) de las microplacas se realizó a 450 nm. Se corrigieron las lecturas de DO restando el valor de lectura del pozo sin antígeno al valor obtenido en el testigo positivo con antígeno. Se estableció el valor de DO de cada muestra en términos porcentuales contra el valor corregido del testigo positivo (S/P%). Siguiendo las instrucciones del fabricante del paquete, las muestras con valor S/P% igual o menor a 40% fueron consideradas negativas, con S/P% entre 40 y 50% sospechosas, y con valores S/P% iguales o mayores a 50% positivas.

## **Contrainmunolectroforesis**

Mediante pruebas de contrainmunolectroforesis (CIE) se constató la presencia de inmunoglobulinas en las muestras de suero fetal. Se preparó un suero hiperinmune contra inmunoglobulinas caprinas en conejo. En un gel de agarosa al 1% con amortiguador de barbituratos pH 8.2, se cortó un canal en medio del gel, con dos perforaciones laterales. En las perforaciones laterales se colocaron 50 µl de suero fetal. El gel se instaló en una cámara de electroforesis y se corrió a 70 voltios durante 45 minutos, posteriormente, se agregaron 300 µl del suero hiperinmune de conejo en el canal central y se incubó a TA en cámara húmeda de 48 a 72 horas.

## **Resultados**

Los fetos obtenidos presentaron edades de 50 a 145 días de gestación.

Se detectaron nueve fetos seropositivos (7.5%) y cinco sospechosos (4.2%) a LvPR, cinco fueron ca-

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the other three seropositive sera and in the five suspicious sera.

## Discussion

Considering the way in which fetal serum samples were obtained, contamination of samples with maternal blood was highly unlikely. Ruminant fetuses and newborn ruminants are agammaglobulinemic, since the placenta of these species is impermeable to the passage of maternal immunoglobulins;<sup>20</sup> therefore, the presence of antibodies against SRLV is not only an indicator of the fetal response, but also an unequivocal indicator of fetal infection with these viruses. The results obtained in positive and suspicious fetuses demonstrate that they were capable of generating antibodies against SRLV, at least since the beginning of the second stage of gestation, 60 days. Due to the lack of stimuli and maturity, in a sterile uterine environment, the fetal immune system of ruminants in development and differentiation, as well as that of the other species, develops mainly responses of the IgM class, and in a lesser extent of the IgG class.<sup>19, 21</sup> The characteristic IgM banding observed in CIE tests supports this possibility. It is likely that positive and suspicious sera that did not show this band in CIE (8:14) had low gamma globulin concentrations, insufficient to be detected by this technique.

According to the manufacturer, the ELISA test used mainly detects antibodies of the IgG class, therefore, the possibility should be raised that the number of positive fetuses may have been greater than that demonstrated, and that these were not detected by the technique used. This observation is particularly valid for caprine fetuses, considering the wide distribution of the disease in Mexico, while on the contrary, the number of positive and suspicious fetuses is noticeable, considering that MV appears to be a disease with a lower prevalence in Mexico, although in this case it is also possible that commercial flocks, which have imported livestock from the USA and Canada without

**CUADRO 1**

Resultados de la respuesta serológica con ELISA, de fetos ovinos y caprinos, a lentivirus de pequeños rumiantes

Ovine and caprine fetuses ELISA response to small ruminant lentiviruses

	<i>Caprine fetus</i>	<i>Ovine fetus</i>	<i>Fetus age (days)</i>
Positives	5	4	80-100
Negatives	46	59	50-145
Suspected	3	2	57-63

prinos de aproximadamente 80 días y cuatro fueron ovinos de entre 90 y 100 días de gestación. Los fetos sospechosos fueron de menor edad, aproximadamente 60 días de gestación, tres de origen caprino y dos ovinos (Cuadro 1).

En CIE, seis de los sueros positivos formaron una banda de precipitación característica de IgM, que no se presentó en los otros tres seropositivos y los cinco sospechosos.

## Discusión

Considerando la forma de obtención de las muestras de suero fetal, su contaminación con sangre materna resulta altamente improbable. Los fetos y recién nacidos de los rumiantes son agammaglobulinémicos, al ser la placenta de estas especies impermeable al paso de inmunoglobulinas maternas,<sup>20</sup> por lo que la presencia de anticuerpos contra LvPR es además de indicador de la respuesta fetal, indicador inequívoco de la infección de los fetos por estos virus. Los resultados obtenidos en fetos positivos y sospechosos demuestran que fueron capaces de generar anticuerpos contra LvPR a partir, al menos, del inicio del segundo tercio de la gestación, 60 días. Por la falta de estímulos y de madurez en un ambiente uterino estéril, el sistema inmune fetal de los rumiantes en desarrollo y diferenciación, igual que el de las demás especies, desarrolla principalmente respuestas de la clase IgM, y en menor medida, IgG.<sup>19, 21</sup> El bandeo característico de IgM, observado en las pruebas de CIE, soporta esta posibilidad. Es posible que los sueros positivos y sospechosos que no presentaron esta banda en CIE (8:14) tuvieran bajas concentraciones de gammaglobulinas, insuficientes para ser detectadas por esta técnica.

De acuerdo con el fabricante, ELISA detecta principalmente anticuerpos de la clase IgG, por lo que se debe plantear la posibilidad de que el número de fetos positivos fuera mayor al demostrado, y que éstos no fueron detectados por la técnica utilizada. Esta observación es particularmente válida para los fetos de origen caprino, considerando la amplia distribución de la enfermedad en México. Por el contrario, resulta llamativa la cantidad de fetos ovinos positivos y sospechosos en atención a que MV parece ser una enfermedad de menor prevalencia en México, aunque también en este caso, es posible que los rebaños del ganado importado sin control por empresas de Estados Unidos de América y Canadá, puedan presentar prevalencias equivalentes a las demostradas en aquellos países.

No se encontraron informes previos sobre la demostración de anticuerpos contra LvPR en fetos ovinos y caprinos. Existe información que indica la posibilidad de que el feto pueda ser infectado con LvPR durante la



any control, may have prevalences equivalent to those demonstrated in these countries.

No previous reports were found on the demonstration of antibodies against SRLV in ovine or caprine fetuses. There is information indicating the possibility that the fetus may be infected with SRLV during gestation.<sup>6</sup> Viral infection of the fetus may cause alterations that trigger fetal resorption or abortion, according to the moment of gestation in which it occurs and to the type of virus involved; when this does not happen, the fetus may be immunotolerant, as seen in bovine viral diarrhoea. Fetal infection in the early stages of gestation leads to viral antigens being recognized as the fetus' own antigens, and the animals develop serious disease when they are born, which is usually fatal.<sup>30</sup> Due to the characteristics of this work, the time of product infection could not be established, and the possibility that tolerance phenomena are established in early SRLV infection should not be ruled out. The antibodies demonstrated in caprine fetuses may, however, be involved in the pathogenesis of encephalic diseases that occur in goats of two to four months of age, associated with the lesions directly induced by viral presence.

The results of this work coincide regarding the proportion of reactive fetuses, with the viral presence observed by PCR in lambs without colostrum intake in flocks with endemic MV,<sup>13,14</sup> and challenge the control strategies based on the lack of direct colostrum feeding of offspring from mothers proven to be positive to SRLV, which imply high labour costs and newborn losses due to starvation. In any event, artificial colostrum or bovine or pasteurized colostrum feeding strategies and the separation of flocks between positive and negative mothers,<sup>7,8,14</sup> may be used until prevalence of the disease is overthrown in the flock, in order to proceed to the elimination of all reactive females as soon as the economic conditions for replacement allow it.

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