

Early Detection of goats infected with Lentivirus Small Ruminant virus by ELISA assay

Detecção precoce de caprinos infectados com Lentivirus de pequenos ruminantes através do ensaio ELISA.

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Abstract

Many established protocols are available to produce antigens for ELISA tests but expensive equipments, the purification methods or recombinant technologies are not always available to veterinary diagnostic laboratories. The aim of this work was to develop an alternative ELISA test to detect antibodies against Lentivirus Small Ruminant Virus (*Caprine arthritis-encephalitis virus-CAEV*) in goats, simple to elaborate with a low cost of production. The antigen was obtained from a whole cellular lysates of goat synovial membrane cells (GSMC) infected with CAEV and treated with SDS 0,1%. The whole-viral antigen was capable of detecting antibodies against several viral proteins including the p28 kDa (viral capsid), the p33 kDa, the p44 kDa (capsid precursors) and p97 kDa (transmembrane glycoprotein). The comparison with others assays as indirect immunofluorescence showed a high correlation with the ELISA results and the polimerase chain reaction (PCR) demonstrated that most of the goat seropositives by ELISA, had proviral DNA in the milk cells. In conclusion, the viral antigen obtained from GSMC was simple to elaborate, low cost of production. The ELISA test was capable of detecting antibodies against CAEV, to detect early infection.

Keywords: Lentiviruses, Ovine-Caprine. Enzyme-Linked Immunosorbent Assay. Diagnosis.

Resumo

Vários são os protocolos disponíveis para a produção de antígeno para testes de ELISA, entretanto equipamentos caros, métodos de purificação ou tecnologias recombinantes não estão sempre disponíveis para os laboratórios de diagnóstico veterinário. O objetivo deste trabalho foi desenvolver um teste de ELISA para detectar anticorpos contra o Lentivirus de pequenos ruminantes (Artrite encefalite caprina-CAEV) em caprinos. O antígeno foi obtido de lisados de cultivo de células de membrana sinovial caprina (GSMC) infectados com CAEV e tratado com SDS 0,1% foi capaz de detectar anticorpos contra várias proteínas virais, incluindo a p28 (cápside viral), p33, p44 (proteínas precursoras da cápside) e p97 kDa (glicoproteína de transmembrana). A comparação com outros ensaios como a Imunofluorescência indirecta mostraram uma alta correlação e a reação em cadeia da polimerase mostrou que a maioria das cabras assintomáticas soropositivas por ELISA, tinham DNA viral nas células do leite. Em conclusão, este ensaio de ELISA com antígeno viral obtido a partir de GSMC, de forma simples, com um baixo custo de produção, foi capaz de detectar forma precoce a infecção por vírus CAEV em cabras assintomáticas.

Palavras-chave: Lentivirus Ovinos-Caprinos. Ensaio de Imunoabsorção Enzimática. Diagnóstico.

INTRODUCTION

Caprine arthritis-encephalitis (CAE) is a progressive and debilitating disease caused by a *Caprine arthritis-encephalitis virus* (CAEV) nowadays referred to as a small ruminant lentivirus (SRLV) (GOFF, 2006; CALLADO et al., 2001). This disease has an economic

impact on livestock, especially dairy goats; CAEV is transmitted from dam to progeny by the ingestion of colostrum or milk; however contact transmission between goats infected also occur (BLACKLAWS et al., 2004).

The virus infects monocytes and induces chronic progressive arthritis, indurative mastitis, pneumonia in goat adults and leuko-encephalomyelitis commonly in goat kids but the clinical disease are not evident for several years after infection (NARAYAN et al., 1983).

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The infection is characterized by late seroconversion or intermittent seropositivity and seronegativity. There are several serological tests that can be used to detect infection, such as enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence (IFI) as well as western blot (WB) and agar gel immunodiffusion (AGID). For regulatory purposes, the Organization of International Epizootics (OIE, 2004) recommends using AGID test for the serological diagnosis of SRLV. However, there are immunoenzymatic assays (i.e., ELISA) that are more sensitive and faster than AGID but the widespread use of these immunoenzymatic assays is limited. Many established protocols are available to produce antigens for ELISA tests but purification steps or recombinant technologies are not always available for veterinary diagnostic laboratories. In an effort to improve the diagnosis of CAEV infection and minimize the possible economic burden associated with production of the ELISA tests, we have developed an ELISA assay, which uses whole cellular lysates from infected primary GSMC.

MATERIALS AND METHODS

Antigen

The viral antigen for indirect ELISA test was obtained from primary GSMC infected with CAEV (Decente strain; Tigre et al., 2006). When extensive cytopathic effect was seen (usually 18-21 days), the virus-containing cells and supernatant were frozen/unfrozen and clarified by centrifugation (10.000 x g 30 minutes at 10°C). The sediment obtained was treated with SDS 0,1 % (Simard et al., 2001) during 10 minutes, submitted to centrifugation (10.000 x g 15 minutes) to collect the supernatant and stored at -70 °C until use. Mock-infected antigen was prepared as described above from day 21 mock-infected GSMC.

Serum samples

The samples to carry on this work were obtained from 64 symptomless females and males goats (1 to 4 years of age) from flocks without clinical signs of CAEV. Serum samples were collected (n=64) for antibody detection, and milk samples were collected (n=40) for DNA extraction from milk cells to detect DNA provirus.

1.3 Whole-viral antigen ELISA test

The ELISA test was performed as described by Torres et al., (2009) with some modifications. Briefly, a microtiter plates (NUNC-Maxisorp™) were coated with whole-viral antigen and mock-infected antigen (3.0 µg/well) in alternate rows and incubated overnight at 4 °C. A blocking solution consisting of 5% skim powder milk in 0.01 M phosphate-buffered saline (PBS) was added to each well and incubated for 1 h at 37 °C. Wells were washed 5 times with PBS containing 0.05% Tween 20 (PBS-T). Serum samples were added to each well (whole-viral antigen and mock-infected antigen), diluted (1:100) in PBS-skim milk (0.5%) buffer with 0.3% Tween 20. The plates were incubated overnight at 4 °C. After that, the

wells were washed as mentioned above and it was added a rabbit anti-goat immunoglobulin horseradish peroxidase (HRPO) conjugated antibody (Sigma Chemicals Co.). The plates were incubated for 1 h at 37 °C. For colour development, 3,3',5,5' tetramethylbenzidine (TMB, Sigma Chemicals Co.) substrate was added to each well. The absorbance values were read at 655 nm. The optimal ELISA cut-off was determined by a *receiver operating characteristic curve* (MedCalc statistical software). The cutoff values were established at the mean optical density at 655 nm (OD_{655}) of the 40 negative samples tested on the positive antigen-coated wells. A sample was declared positive if its OD_{655} was more than the cutoff value of 0,209.

AGID test

AGID commercial kit (Biovetech®, Brazil) was used according to the manufacturer's instructions. The test used a specific CAEV antigen, the p28 capsid protein.

IFI test

The IFI test was performed in GSMC cultivated on glass slides and infected with CAEV. The slides were fixed in cold acetone for 10 min at -20°C, air dried and incubated for 1 h at 37°C with negative or positives serum samples (n=11) diluted 1:10 in PBS. After that, the slides were rinsed with PBS 3 times (10 minutes each); incubated with anti-goat FITC conjugated (1:50; Sigma Chemicals Co.) for 1 h at 37°C and rinsed with PBS as mentioned before. The final rinse was done with Evans blue (0.01%) and the slides were examined with a fluorescence microscope. The presence of cytoplasmic apple-green fluorescence indicates detection of CAEV-infected cells.

Western blot

Western-blot was carried out following conventional techniques. Briefly, the whole-virus antigen or mock-infected antigen GSMC were dissolved in denaturing buffer (30 mM TRIS [pH 6.8], 2% SDS, 10% 2-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue) and heated at 95°C for 3 min. The samples were loaded in the SDS-PAGE 15% to submit to protein electrophoresis and transferred to a nitrocellulose membrane by electroblotting (110 V, 1 h). For WB technique, an AGID CAEV-positive reference serum, positive serum samples (n=10) and monoclonal antibody against CAEV (Brandão et al., 2009) were diluted 1:10 and incubated overnight at 4 °C with the membrane strips. After several rinses with PBS-T, secondary rabbit anti-goat or anti-mouse HRPO-conjugate antibody (Sigma Chemicals Co.) (1:1000) were added and incubated for 1 h at 37 °C. Then, the strips were washed again with PBS-T and the substrate 3,3 diamino benzidine (DAB) 10 mg/ml in presence of H_2O_2 , was added for colour development.

1.7 Double-nested PCR

The proviral DNA amplification by double-nested PCR was carried on with milk samples (30 ml) (Tigre et al., 2006). The samples were spun at 3000 x *g* for 15 minutes at 10°C and the fat and cream layer were discarded. The excess fat from cells was removed by sequential washing and centrifugation in PBS. The cells were resuspended (0.5 ml) in PBS and DNA was extracted using the QIAamp DNA Mini kit (Qiagen, Inc. UK). Double-nested PCR was carried out using two pairs of oligonucleotide primers to amplify *pol* and *env* sequences of CAEV as described previously by Travassos et al. 1999. The outer primers were 5374 and 5376 for *pol* sequence and 99001 and 5086 for *env* sequence. The inner primers were 5375 and 5377 for *pol* sequence and 99006 and 99008 for *env* sequence. The sequences of the primers used to amplify the subgenomic fragments of CAEV are numbered according to the published strain reference CAEV Cork (SALTARELLI et al., 1990). Amplification was carried out in a thermocycler gene Amp OCR System 2400 (Perkin Elmer) according to Travassos et al. (1999) and the PCR products were visualized in 1,2 % agarose gels by staining with ethidium bromide and photographed with a Kodak Polaroid Camera.

Data Analysis

The sensitivity, specificity, predictive value and *k* coefficient of agreement were calculated using a *Statistics Packing Social Science (SPSS)* program for Windows 14.0. The *k* coefficient was calculated considering 0 equal to no agreement and 1 indicate perfect agreement

RESULTS

As shown in Table 1, a whole-virus antigen ELISA test showed greater sensitivity (91,1%) than the AGID test. The number of positive sera detected by ELISA was higher (44/64 or 68.75%) than those detected by AGID (36/64 or 56.22%). The ELISA showed a high sensitivity and fair agreement with the AGID according to *kappa* value ($\hat{\epsilon} = 0.21$).

TABLE 1 - Sensitivity , specificity and *k* coefficient of the ELISA test in comparison to AGID assay in goat serum samples^a

| ELISA results | IDGA results | | |
|---------------|--------------|----------|----|
| | Positive | Negative | |
| Positive | 34 | 10 | |
| Negative | 2 | 18 | |
| TOTAL | 36 | 28 | 64 |

^a n=64 ELISA sensitivity = $[34 / (34+2)] \times 100 = 91,9\%$; ELISA specificity: $[18 / (10+18)] \times 100 = 64,2\%$. Positive Predictive Value : 77% ; Negative predictive value: 90% ; *k* coefficient of agreement : 0,21 (fair agreement).

The WB (Fig 1) shows that whole-viral antigen was successfully able to detect antibodies against several proteins. Seropositive goat sera reacted strongly against the viral p28 kDa protein (Fig. 1, lanes A, B, C) and also demonstrated reactivity with the p33 kDa, p44 kDa and p97 kDa proteins. The comparative results between WB and ELISA showed that 10/13 (76.9%) were positive in both tests and 3/13 (23,0%) were positive only for WB. There was moderate agreement with ELISA according to *kappa* value ($\hat{\epsilon} = 0.55$).

Of the eleven goat serum samples, ten showed a positive reaction in IFI, demonstrated by green fluorescence in the cytoplasm of infected cells. The pattern of reaction by IFI of goat serum samples is shown in Figure 2 A,B. The positive control (rabbit hyper-immune serum anti-CAEV) showed a clear and strong cytoplasmic fluorescence (Fig 2, C). The comparative results with ELISA showed a high correlation with these two techniques where 9/11 (81.8%) serum samples were positive in both tests and 1/11 (9.0%) was negative. For only one serum sample, no antibodies were detected by ELISA test, but it was IFI positive. There was substantial agreement between IFI and ELISA according to the *kappa* value ($\hat{\epsilon} = 0.62$).

Double-nested PCR revealed the presence of CAEV in milk cells. Figure 3 shows the amplification of the *pol* (175 bp) and *env* (350 bp) viral genes from proviral DNA of milk cells. ELISA and PCR comparisons are listed in Table 2. As is shown, *pol* and *env* genes were detected in 72% milk samples from goat seropositives by ELISA test. However, it was not possible detected proviral DNA in seven milk samples from seropositive goats. On the other hand, *pol* and *env* viral genes were detected in 53% of milk samples from seronegative goats. The *kappa* value of 0,15 ($\hat{\epsilon} = 0.15$) suggest slight agreement between double-nested PCR and ELISA.

DISCUSSION

The low levels of antibodies, late seroconversion as well as intermittent seropositive and seronegative reactions makes serological diagnosis of infected animals challenging (CLAVIJO and THORSEN, 1995; HANSON et al., 1996). Because SRLV infection induce low immunological response in infected animals, highly sensitive serological techniques involving highly efficient antigen-antibody interactions are required for proper detection of viral infection. Therefore, immunoenzymatic assays (ELISA) that require only one epitope interaction per antibody are a better choice to those that demand the interaction of many epitopes per antibody (AGID; CELER et al., 1998; REISCHAK et al., 2002).

In this work, an indirect ELISA test performed using whole cellular lysates from infected GSMC treated with SDS showed a higher sensitivity than AGID as expected (LARA et al., 2002; MOREIRA et al., 2005; BRINKHOF et al., 2007). The whole-viral antigen ELISA test detected early seroconversion in healthy and

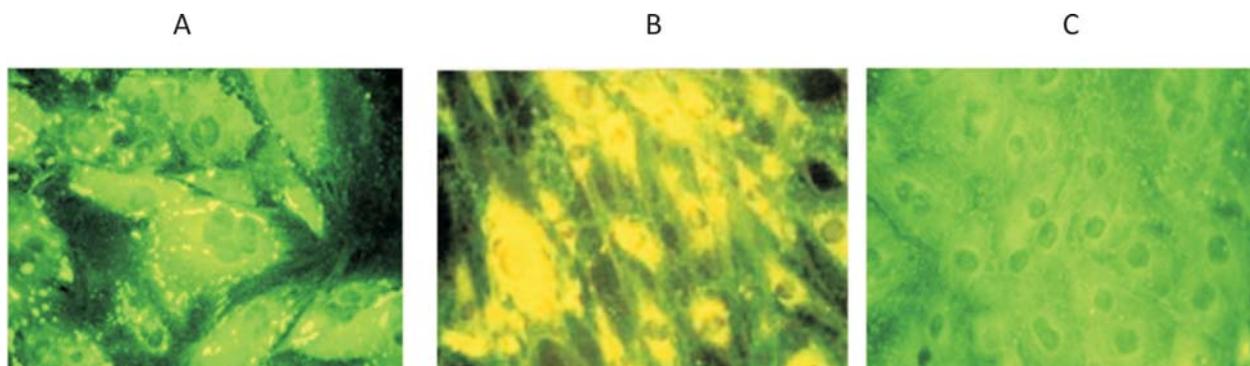


Figure 1- Indirect Immunofluorescence of CAEV in GSMC.

The images represent: (A): GSMC-infected; positive goat serum; (B) GSMC-infected; rabbit hyperimmune anti-CAEV; (C) GSMC non-infected, positive goat serum. The presence of cytoplasmic apple-green fluorescence indicates detection of CAEV infected cell (400x).

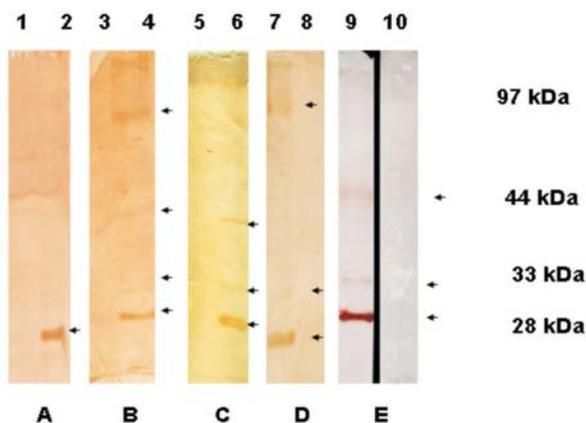


Figure 2 - Western-blot analysis

Lanes 1-3-5-8-10 Mock-infected antigen; Lanes 2-4-6-7-9 Whole-viral antigen ELISA (A) Reference positive serum AGID; (B) (C) e (D) Positive goat sera; (E) Monoclonal antibody against CAEV. The arrows indicate a position of the viral proteins according to the molecular weight markers: p28 kDa (capsid protein), p33 kDa and p44kDa (capsid protein precursors), p97 kDa (transmembrane glycoprotein precursor).

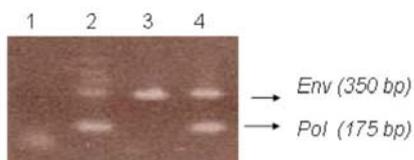


Figure 3 - Double-nested PCR in milk sample

DNA amplification of fragments of *pol* and *env* genes of CAEV provirus. Lane 1: Negative control reaction, Lanes 2 and 4: Positive milk samples, *pol* (175 bp) and *env* (350 bp) products; Lane 3: Positive milk sample, *env* (350 bp)

infected goats from several flocks with a low prevalence of the disease. In general, the low prevalence of the infection makes the diagnosis even more challenging and can lead to false-negative results. Diagnostic sensitivity and early detection have an epidemiologically significant importance as both are

TABLE 2 - Sensitivity, Specificity and *k* coefficient of the ELISA test in comparison to PCR.

| | PCR results ^a | | |
|----------------------------|--------------------------|----------|----|
| | Positive | Negative | |
| ELISA results ^b | | | |
| Positive | 18 | 7 | |
| Negative | 8 | 7 | |
| TOTAL | 24 | 14 | 40 |

^a Milk samples, n= 40; ^b Serum samples, n= 40 ; ELISA Sensitivity = $[18 / (18+8)] \times 100 = 69,2 \%$ ELISA Specificity: $[7 / (7+7)] \times 100 = 50\%$. Positive Predictive Value : 72%; Negative predictive value: 46,6 % . *k* coefficient of agreement : 0,15 (slight agreement).

needed to avoid the persistence of healthy viral carriers animals (false-negatives) in the flocks. In the case of CAEV, a false-negative result is more harmful than a false-positive; therefore, it is very important to use highly sensitive tests with a high negative predictive value to reduce this possibility. When compared the whole-viral antigen ELISA with the AGID test, the negative predictive value of the ELISA test was higher.

WB demonstrated that the immune response of the seropositive goats was principally targeted towards the capsid protein (p28 kDa), the most numerous protein (60 copies/virion) of the virus (GOOF, 2006; CHEEVERS et al., 2000). Antibodies against p28 arise at the beginning stages of infection, but levels decline as clinical manifestations appear. At that moment, antibodies towards the envelope glycoproteins begin to rise (BERTONI et al., 2000). Precursor capsid proteins, viral glycoproteins and their precursors are antigenic as well, and the detection of antibodies against these antigenic proteins will increase the sensitivity of the test. As was demonstrated here, the whole-viral antigen detected antibodies to many viral proteins including the p28 kDa (viral capsid), the p33 kDa, the p44 kDa (capsid protein precursors) and p97 kDa (transmembrane glycoprotein) (CHEEVERS et al., 1988;

SCHOBORG et al., 2002). The ability to detect antibodies directed against diverse viral proteins will increase the chance of detecting infected animals in any clinical phase of the illness (GREGO et al., 2002; ANDRES et al., 2005; BRINKHOF, et al., 2010). Brinkhof et al., (2010) reinforced the concept that the sensitivity of an ELISA test for SRLV is correlated with the stage of the infection therefore an ELISA test in which the antigen is a combination of SRLV glycoproteins and SRLV core proteins will demonstrate the highest efficiency.

Milk is the primary route of spread for CAEV and it compromises the overall health of the flock because goat kids are earlier exposed to CAEV. Here, most of the goat ELISA seropositives had proviral DNA in the milk cells, confirming as expected, that they were healthy carriers of the virus. Most of the time, seropositive goats are apparently healthy without clinical manifestations; therefore, these animals must be detected as early as possible to prevent viral dissemination in the flock. In certain seropositive animals, proviral DNA was undetectable by PCR. Supported by other published reports, this could be a consequence of low viral DNA copy number in the cellular genome, an insufficient number of infected cells to detect the virus, or low viral loading in blood from healthy animals (RUTKOSKI et al., 2001). Additionally, this work found that some seronegative individuals had proviral DNA in their milk cells. These results are in agreement with those observed by Torres et al. (2009) and Tigre et al. (2006). The risk of the late seroconversion always exists and is a characteristic of lentiviruses in general, and it is therefore recommended to double-check the diagnosis of the SRLV with multiple different assays. This scheme is routinely used in the serological diagnosis of another severe lentivirus, *Human immunodeficiency virus*, where complementary serological tests such as WB or IFI confirm the diagnosis for those sera that are classified as reactive or indeterminate (REISCHAK et al., 2002). These serological assays, WB and IFI, were performed in parallel to ELISA test and showed a high correlation with the ELISA results.

In conclusion, the whole-viral antigen obtained from GSMC was simple to elaborate and has a low cost of production. The ELISA test was capable of detecting antibodies against CAEV, sensitivity superior to that of AGID and ability to detect early infection. However, before it can be used in control program, it will be necessary to continue the studies in more flocks and serum samples.

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