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First isolation and nucleotide comparison of the *gag* gene of the caprine arthritis encephalitis virus circulating in naturally infected goats from Argentina

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Abstract
Caprine arthritis encephalitis virus (CAEV) has been reported in different countries worldwide, based on serological and molecular detection. In Argentina, the prevalence of CAEV infections is increasing, with goats showing symptoms associated mostly with cachexia and arthritis. Although in Argentina the virus has been detected by serology, it has never been isolated or characterized. Thus, the objectives of this work were to isolate and analyze the nucleotide sequences of the *gag* gene of Argentine CAEV strains and compare them with those of other SRLVs previously reported. Nucleotide sequence comparison showed homology with CAEV-Co, the CAEV prototype. Phylogenetic analyses showed that the Argentine strains clustered with genotype B, subtype B1. Because the molecular characterization of the *gag* region is suitable for phylogenetic studies and may be applied to monitor the control of SRLV, molecularly characterizing the Argentine CAEV strains may help develop a proper plan of eradication of CAEV infections.
Keywords: CAEV isolate, Caprine arthritis encephalitis virus, *gag* gene.

Introduction

Caprine arthritis encephalitis virus (CAEV) infections in goats have been reported in different countries around the world, based on serology and molecular detection. In Argentina, the last report on the CAEV epidemiological status was based on an enzyme-linked-immunosorbent assay (ELISA) of sera collected from 2010 to 2011. That study evidenced an increase in farm goats positive for CAEV from 19.4% in 2010 to 21.5% in 2011, while the prevalence of positive animals in 2011 was 3.86% (Trezeguet *et al*., 2013).

CAEV infects macrophages and dendritic cells, causing immune-mediated lesions in a variety of organs and leading to lymphocyte accumulation (Blacklaws *et al*., 1994). Around 30 to 40% of infected animals develop clinical symptoms (Cheevers *et al*., 1988), associated with interstitial pneumonia, mastitis, encephalitis, and arthritis (Houwers *et al*., 1988; Narayan and Clements, 1989). Clinical and subclinical mastitis can lead to substantial economic losses in the goat industry (Leitner *et al*., 2004, 2010). However, the most common clinical manifestation of CAEV infections in goats is the arthritic form (Pugh, 2002).

CAEV is genetically and antigenically closely related to the visna-maedi virus (VMV), also known as the ovine progressive pneumonia virus. Both CAEV and VMV are grouped into small ruminant lentiviruses (SRLVs), where cross-species transmission can cause lentiviral diseases in goats and sheep (Pasick, 1998; Shah *et al*., 2004; Pisoni *et al*., 2007).

The genome of SRLVs has the typical proviral genomic organization of the genus *lentivirus*, family Retroviridae and subfamily Orthoretrovirinae (www.ictvonline.org). It consists of two identical single strands of the RNA (+) subunit, and contains the *gag, pol* and *env* structural genes and the *tat, rev,* and *vif* regulatory genes, flanked by non-coding long terminal repeat regions (Gifford, 2012).

The SRLVs are divided into five principal groups (A to E) according to their sequences. Genotypes A, B and E may be further distributed into different subtypes, differing in 15% to 27% of their nucleotide sequences. Thus, genotype A has been divided in several subtypes from A1 to A15, genotype B has been divided into three subtypes, B1-B3, and genotype E has only been divided in two subtypes, E1 and E2 (Shah *et al*., 2004). These genetic variations of the virus represent challenges in the SRLV diagnosis implementing the use of native

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proteins from different viral strains obtained from goats or sheep of a given region or country (De Andres *et al*., 2005).

Although the circulation of CAEV and the disease in Argentina have been known for more than ten years, the virus has never been isolated or characterized. Therefore, the aims of this work were to isolate and analyze the nucleotide sequences of the *gag* gene of the CAEV strains circulating in Argentina and compare them with those of other SRLVs previously reported.

Materials and Methods

Saanen goats belonging to five flocks from different geographic areas, located in 'Sierras Pampeanas' and 'Pampa Humeda', from Argentina, with history of CAEV infection were considered for SRLV molecular detection due to the occurrence of clinical symptoms of arthritis and cachexia (Fig. 1A and B).

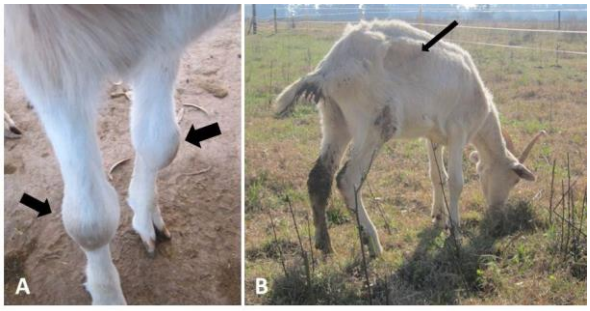

Fig. 1. (A): Saanen goat positive to CAEV with joint carpal arthritis (black arrow). (B): Saanen goat positive to CAEV with cachexia (arrow).

Blood samples with anticoagulant were collected from five goats, each representing its flock, for DNA provirus detection, while synovial fluid from one of them with joint carpal arthritis was collected for virus isolation (Fig. 1A). Peripheral blood leukocytes (PBLs) were separated by centrifugation through Histopaque™ (Sigma–Aldrich) according to the manufacturer's protocols and kept at 4°C until DNA genomic extraction with a commercial kit (DNA Purification Kit, Promega, WI, USA). The synovial fluid from the goat with joint carpal arthritis was extracted with a sterile needle, diluted 1:5 with PBS, filtered with a 0.22-µm pore membrane and co-cultured with CAEV-negative goat synovial membrane (GSM) cells in six-well dishes. One dish was kept without infection as negative control. The cultures were maintained at 37°C in a 5% CO2 atmosphere with minimum essential medium, supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). After several passages, the cultures were stained with Giemsa to display cytopathic effect (CE). For RNA isolation of viral particles from the supernatant of the cell culture with CE, a commercial kit (SV RNA-Isolation System, Kit, Promega, WI, USA) was used.

To amplify the complete *gag* gene (1.3 kb), a hemi-nested PCR was carried out using the primers listed in Table 1. Samples with no amplification were tested by a single round of PCR to amplify a short segment of the *gag* gene (0.645 kb) representing 45% of the gene (Table 1). Primers for the complete *gag* gene were designed by DNAman software according to the CAEV-Cork (CAEV-Co) prototype of CAEV; while the PCR forward primer of the short *gag* region has been reported previously (Valas *et al*., 1997), the reverse primer was designed by the same software according to the CAEV-Co prototype of CAEV and Icelandic 1514 prototype of VMV sequences.

Table 1: List of primers used to amplify the complete and partial *gag* gene in this study.

Primers	Nucleotide sequence 5'-3'	Location
gagF 331-352	AGTAAGGTAAGTGAC TCTGCT	Complete *gag* gene
gagR 1975-1995	AATCCTTGCAGTTTT ATCCTTCC	
gagR 2008-2028	TTATTCCATTTTTCT CCTTCTAC	
gagF 959-979	GCAGGAGGGAGAAGY TGGAA	Partial *gag* gene (45%)
gagR 1608-1628	YCCTTCKGATCCCAC ATCTC	

The reaction conditions of the first and second round of the hemi-nested PCR were: 1 cycle of the initial denaturation at 94 ºC for 5 minutes, 40 cycles of denaturation at 94 ºC for 30 seconds, annealing at 56 ºC for 30 seconds, and extension at 72 ºC for 40 seconds; followed by another extension step at 72 ºC for 10 minutes to complete the reaction. The cycle profile for the short *gag* gene amplification was: 1 cycle at 94 ºC for 5 minutes, 35 cycles of denaturation at 94 ºC for 30 seconds, annealing at 54 ºC for 30 seconds, and extension at 72 ºC for 40 seconds; followed by another extension step at 72 ºC for 7 minutes to complete the reaction. The PCRs were performed in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT, USA) and the PCR reactions carried out with the PCR Master Mix according to the manufacturer's protocols (Promega, WI, USA). The same conditions were used for reverse transcriptase activity with a previous step of 37°C for 60 min.

The amplified products were visualized on 1.5% (w/v) agarose gel and staining with ethidium bromide. Each amplified *gag* gene was subcloned into the pGEM-T easy vector® according to manufacturer's protocol and sent to sequencing in an ABI3130XL Sequencer (Applied Biosystems, USA), Unidad Genómica, INTA Castelar, Argentina. The nucleotide sequences obtained were assembled and analyzed by the Bioedit software and the nucleotide compositions were compared with

other strains reported in GenBank. Genetic homology and the neighbor-joining tree of the nucleotide sequences were assessed using MEGA software version 4.0

Results and Discussion

The first isolate of CAEV in Argentina was obtained from the synovial fluid extracted of the joint carpal arthritis from a goat with clinical symptoms of CAEV infection (Fig. 1). CE coincident with syncytia was observed after two passages using GSM cells (Fig. 2).

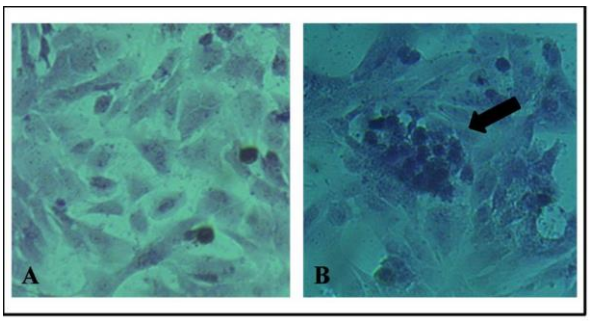

Fig. 2. Culture of goat synovial membrane (GSM) cells. (A): Non-infected cells as negative control. (B): Infected cell with synovial fluid from goat with carpal arthritis; the black arrow shows cytopathic effect (CE) coincident with syncytium after two passages (25X).

The presence of viral particles in the supernatant of the cell culture was quickly confirmed by reverse transcriptase activity by hemi-nested PCR amplifying the complete *gag* gene of 1.3 kb. In contrast, the amplification of the CAEV from the remaining four goats was obtained from DNA genomic provirus detected in PBLs.

Because it was not possible to amplify the complete *gag* gene of the virus in these goats, probably due to the variability in nucleotide composition, we amplified a product of 0.645 kb corresponding to 45 % of the *gag* gene. The variability in the nucleotide sequences characteristic of lentiviruses may result in a high rate of mutation, capable of forming many different strains or quasispecies (Santry *et al*., 2013). Thus, new primers should be designed to succeed in the amplification of the strains circulating in Argentina.

Because a *gag* region is suitable for phylogenetic studies and may be applied to monitor SRLV eradication programs (Santry *et al*., 2013), the five nucleotide sequences obtained were aligned and compared with the SRLV sequences reported in GenBank. The percentage of major homology found between the complete and short region of the *gag* gene showed an average of 90.5% of identity compared with CAEV-Co sequences. Moreover, the nucleotide composition of the Argentine strains showed a range of 86 to 92% identity compared to each other.

In addition, the phylogenetic trees were built using alignments of the complete (Fig. 3A) and partial *gag* genes (Fig. 3B).

Our analysis was carried out by neighbor-joining algorithm to obtain the differentiation and the branching order of groups to classify the genotypes and subtypes proposed by Shah *et al*. (2004). In both cases, the Argentine strains were clustered together with the isolate classified as genotype B, subtype B1. Regarding the partial *gag* gene, the Argentine strains clustered with the reference strain CAEV-Co isolated from a goat of North America, representative of the prototype caprine lentivirus (Cork *et al*., 1974).

These clades were the same as those for CAEV-Philippines, Gansu-China and a strain reported in Brazil (Ravazzolo *et al*., 2001) (Fig. 3B). Based in these results obtained and compared with the phylogenetic distance in relation to the Icelandic 1514 strain, prototype of VMV (genotype A), the SRLV detected in this work and circulating in Argentina were identified as CAEV.

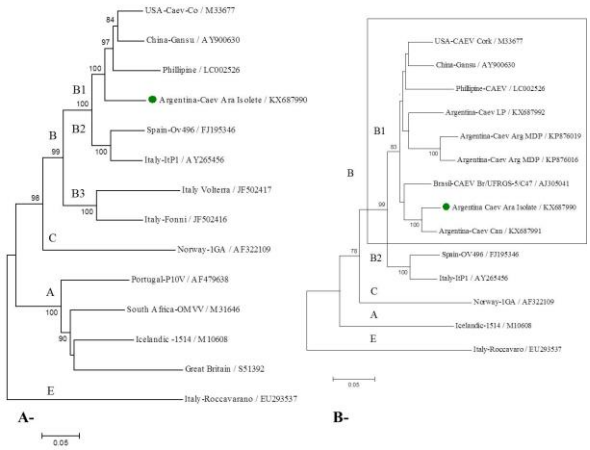

Fig. 3. (A): Neighbor-joining phylogenetic tree using the complete *gag* sequence (1313 bp). (B): Neighbor-joining phylogenetic tree using the partial *gag* sequence (645 bp). Bootstrap values are based on 1000 repetitions. SRLVs representative of each genotype and subtype according to the classification by Shah *et al*. (2004) were used. The sequence of the Argentine isolate is shown with a green circle. Each SRLV sequence is denoted by country and GenBank access number. Sequences of genotype B1 are shown with a box.

Subtype B1 has been reported to have undergone cross-species transmission from goats to sheep (Pisoni *et al*., 2005) and although antibodies of SRLV have also been detected in sheep flocks in Argentina (Trezeguet *et al*., 2013), the virus has never been characterized in this species to determine whether another genotype is circulating.

The success in preventing SRLV infection spread depends largely on early detection of infected animals in the flocks (Ramirez *et al*., 2013). For that, and due to the last report of prevalence in Argentina, studies

should also be conducted to investigate the presence of SRLV strains circulating in sheep, especially in the case where they coexist in the same herd with goats.

In this work, we were able to isolate CAEV for the first time from naturally infected goats circulating in our country. The genetic characterization of the virus is necessary to obtain more information to implement a control method and eradication plan of CAEV infections in Argentina.

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Conflict of interest

The authors declare that there is no conflict of interest.

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