



الكشف عن فيروس التهاب المفاصل والدماغ الماعزي (CAEV) في الدم وأنسجة الجهاز التناسلي عند الماعز في سورية

Detection of Caprine Arthritis-Encephalitis Virus (CAEV) in Blood and Genital Tract Tissues of Goats in Syria

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الملخص

هدفت هذه الدراسة إلى تحديد نسبة الإصابة المصلية لخمج فيروس التهاب المفاصل والدماغ الماعزي (CAEV) في سورية وإلى التقصي عن حالة خمج الدم وأنسجة الجهاز التناسلي عند الماعز المصاب طبيعياً بهذا الفيروس. في البداية، تم التقصي عن الإصابة المصلية بخمج فيروس التهاب المفاصل والدماغ الماعزي في قطعان مختلفة من الماعز السوري وذلك من خلال جمع وتحليل 243 عينة دم ماعزية. في المرحلة الأولى، استُخدم لهذا الغرض تقانة المقايسة المناعية المرتبطة بالأنظيم (ELISA) للكشف عن الأضداد النوعية للفيروس. بعد ذلك استُخدمت تقانة تفاعل البوليميراز المتسلسل (PCR) للكشف عن طليعة الفيروس (CAEV-proviral DNA) في اللمفيات وحيدة النواة لهذه العينات. وفي المرحلة الثانية، استُخدمت تقانة ال-PCR للكشف عن طليعة الفيروس في أنسجة الجهاز التناسلي (الرحم، والقناة الناقلة والمبايض) لـ 47 رأساً من الماعز إيجابية المصل من أصل 176 رأساً من الماعز تم ذبحها في المسلخ. أظهر 47 رأساً من الماعز من أصل 243 (19.34%) أضداداً نوعية لفيروس التهاب المفاصل والدماغ الماعزي، أما نسبة الإصابة المصلية الحقيقية فبلغت 15.7%.

أمكن باستخدام تقانة ال-PCR الكشف عن طليعة الفيروس في خلايا الدم عند 16 رأساً من الماعز من أصل 243 (6.6%) وفي أنسجة الجهاز التناسلي المختلفة (19.15% من الأرحام، و 8.5% من المبايض و 8.5% من القنوات الناقلة) تم جمعها من 47 من أصل 176 رأساً من الماعز إيجابية المصل. لقد ثبت أن وجود طليعة الفيروس في خلايا الدم وأنسجة الجهاز التناسلي كان أعلى وبشكل معنوي ($P < 0.01$) عند الماعز إيجابية المصل.

تُظهر هذه الدراسة وبشكل واضح وجود أضداد نوعية للفيروس وطليعة الفيروس في دم وأنسجة الماعز السوري التناسلية المختلفة.
الكلمات المفتاحية: الماعز، التهاب المفاصل والدماغ الماعزي، الجهاز التناسلي، تفاعل البوليميراز المتسلسل، سورية.

Abstract

The aim of this study was to determine the seroprevalence of caprine arthritis-encephalitis virus (CAEV) infection in Syria, and to investigate the infectious status of blood and genital tract tissues from goats naturally infected with CAEV. Firstly, to investigate the seroprevalence of CAEV infection in different breeds of goats in Syria, blood samples from 243 goats were analyzed. Prevalence of specific antibodies to CAEV was determined using commercially available ELISA test. Then, polymerase chain reaction (PCR) was used to determine whether CAEV proviral-DNA could be detected in the circulating mononuclear cells of the 243 goats. Secondly, PCR was used to detect the proviral DNA of CAEV in the genital tract tissues (uterus, oviducts and ovaries) of 47 seropositive from 176 goats taken in the slaughter-house. Out of the 243 goats investigated, 47 (19.34 %) had specific antibodies against CAEV. Individual goat true seroprevalence to CAEV was 15.7 %. CAEV proviral-DNA was identified by PCR in the blood cells of 16/243 (6.6 %) goats and in the different of genital tract tissues (19.15 % uterus, 8.5 % ovaries and 8.5 % oviducts) from 47/176 seropositive goats. The presence of CAEV proviral-DNA in blood cells and the genital tract tissues was significantly higher ($P < 0.01$) in seropositive goats. This study clearly demonstrates the presence of viral specific antibodies and proviral-DNA in naturally infected Syrian goat blood and in various genital tract tissues.

Keywords: Goats, CAEV, Genital tract, PCR; Syria.

Introduction

Caprine arthritis-encephalitis infection has been detected worldwide, but it is most prevalence in countries where dairy goats are intensively raised (Rowe and East, 1997). This is a very common disease in dairy goats, and it caused by an RNA virus from the Lentivirus genus of the Retroviridae family (Cork et al., 1974). Caprine arthritis-encephalitis virus (CAEV) has a tropism for monocytes and macrophages and causes chronic inflammatory diseases (Narayan et al., 1982, 1983). Most infected goats develop specific antibodies which persist for life (Adams et al., 1980; Crawford et al., 1980), and the detection of antibodies can be taken as proof of the presence of viral infection (Adams et al., 1983; Narayan and Cork, 1990). However, only some of the infected goats, less than 10 % develop clinical disease characterized by chronic persistent arthritis, mastitis (Hard Udder), respiratory distress (Pneumonia) and encephalitis (Crawford and Adams, 1981; Narayan et al., 1983; Zink et al., 1990).

Approximately 30-80 % of dairy goats in USA, Canada, and Europe are infected, compared to 0-10 % in Africa and South America (Phelps and Smith, 1993; Rowe and East, 1997). In the Middle East, CAEV infection was reported in Saudi Arabia, Syria, Jordan and Turkey and the prevalence rates ranged from 0.8 to 12.5% (Alluwaimi et al., 1990; Al-Qudah et al., 2006; Burgu et al., 1994; Giangaspero et al., 1992).

Viral transmission usually occurs horizontally through the ingestion of viral-infected goat milk and/or colostrums (Rowe and East, 1997; Rowe et al., 1991, 1992). Other potential sources of viral transmission include transmission in utero, contact with the vagina of an infected doe during parturition, via saliva or respiratory secretions during mothering, via contact with infected blood, viral contamination of milking equipment, needles, tattooing equipment, and breeding (Adams et al., 1983; Rowe and East, 1997; Rowe et al., 1991, 1992). Suspicion of the presence of CAEV virus in the genital tract of infected goats and the possibility of a vertical or horizontal route of transmission was considered. First, the detection of CAEV-infected cells in postpartum genital secretion of dairy does (Rowe et al., 1999) and second, the detection of CAEV-infected cells in oviductal flushing media recovered during early embryo collection from infected goats (Fieni et al., 2002).

CAEV infection causes economic losses due to reproductive failure, lowered milk production, reduction in lactation length and an increased incidence of inter-current diseases (Greenwood et al., 1995).

A limited number of studies is available on prevalence of CAEV infection among goat population in Syria because this disease of CAEV has not been reported to occur in Syria before 1992. Syria is located between 35° 00" N 38° 00" E with an annual temperature ranging from -4 to 32°C. The humidity varies from 15 to 100%. The population of sheep and goats in Syria is 22865366, 1561265, respectively (Statistics of Ministry of Agriculture, 2007). Small Ruminants population in Syria increased directly after the second Gulf War due to illegal movement of animals from Iraq. Vaccinations against the major

infectious diseases are carried by the government and are free of charge. Vaccination against CAEV is not practiced.

Previous investigation in Syria, using agar immuno-diffusion test indicated a prevalence of 12.5 % (Giangaspero *et al.*, 1992). However, this survey was conducted on a target population and only analysed 72 samples collected from 4 flocks in different locations of north-west Syria. No report is available on the prevalence of CAEV infection among goat population in other regions (in the South and Middle areas). In these areas, goats are the most important source of meat and milk for the rural population, and Shami and local goats are two indigenous breeds reared in the regions.

The aim of this study was to investigate the seroprevalence of caprine arthritis-encephalitis virus (CAEV) infection in different breeds of goats in Syria and to determine whether CAEV proviral-DNA could be detected in the circulating mononuclear cells and in the genital tract tissues (ovaries, oviducts and uterus) of seropositive goats.

Materials and methods

1. Study location:

Firstly, to determine the prevalence of CAEV in the farms, blood samples were collected from 243 Shami and local goats from nine farms in south and middle regions of Syria. Herds and animals within each herd were selected by random sampling. Secondly, to detect the presence of CAEV in genital tract tissues, blood samples and genital tract tissues were taken in the slaughter-house from 176 Shami and local goats.

2. Sample collection:

In the farms, whole blood samples (8 ml) were taken by jugular venipuncture into anticoagulant (lithium heparin) from each goat (243goats). In the abattoir, blood and samples of uterus, oviducts and ovaries from (176) goats were collected and stored at -70 °C until ELISA and PCR testing.

3. Preparation of the samples for ELISA and PCR:

3.1. Blood:

The whole blood, collected in anticoagulant, was centrifuged at 1900 x g for 30 min at room temperature using Ficoll density-gradient centrifugation. At the end of this first phase 2 ml of serum was stored at -20 °C until testing by ELISA and leucocytes were recovered from the buffy coat, washed in sterile PBS pH 7.2, and centrifuged for 5 min at 700 x g. This washing step was repeated three times. The supernatant was discarded and the cell pellet stored at -80 °C until subsequent DNA extraction. DNA was extracted from the leucocytes using a «QIAamp Tissue kit®» (Qiagen, Courtaboeuf, France), in accordance with the manufacturer's instructions. The samples were then stored at -20 °C, awaiting PCR analysis.

3.2. Tissues:

After thawing, DNA was extracted from the different genital tract tissues (uterus, oviducts and ovaries) using a «QIAamp Tissue kit®» (Qiagen, Courtaboeuf, France). The samples were then stored at -20 °C, awaiting PCR analysis.

4. Sample analysis by ELISA:

All collected sera were evaluated for anti-CAEV surface envelope (SU) antibodies using a commercially available ELISA CHEKIT CAEV/MVV kit (IDEXX Laboratories B.V.-Koolhovenlaam, NEDERLAND). The test was performed in line with the manufacturer's instructions. The kit includes standard positive and negative control goat sera. Optical density of a sample was determined with the ICN Flow Titertek Multiscan (Labsystems, Espoo, Finland), setting the wave length at 450 nm. Determination of seropositive and seronegative sera was calculated as suggested in the kit's leaflet. The sensitivity and specificity of this ELISA kit was previously investigated and were 100 and 95.6 %, respectively (Saman *et al.*, 1999).

5. Procedure for nested-PCR (polymerase chain reaction):

The nested-PCR technique, as described previously by Barlough *et al.*, (1994) was used to detect the presence of CAEV proviral-DNA in the blood and genital tract tissue samples. Two rounds of PCR amplification were used to detect the gag sequence of the CAEV genome.

In the first round, viral detection was performed via the amplification of a fragment of proviral-DNA, located between nucleotide 393 and nucleotide 1291, using external primers GAG EX5 (5'- GAA GTG TTG CTG CGA GAG GTC

TTG -3') and GAG EX3 (5'- TGC CTG ATC CAT GTT AGC TTG TGC -3'). This round was immediately followed by a second round, amplifying the fragment located between nucleotide 524 and nucleotide 1036, using internal primers GAG IN5 (5'- GATAGA GAC ATG GCG AGG CAA GT-3') and GAG IN3 (5'- GAG GCC ATG CTG CAT TGC TAC TGT -3'). Oligonucleotide primers specific to the fourth exon of the human β -actin gene were used as an internal control, for the integrity of the DNA lysates: external-ES30 (5'- TCA TGT TTG AGA CCT TCA ACA CCC CAG -3') and ES32 (5'- CCA GGG GAA GGC TTG AAG AGT GCC -30) were used for the first round, and internal-ES31 (50- CCC CAG CCA TGT ACG TTG CTA TCC -3') and ES33 (5'- GCC TCA GGG CAG CGG AAC CGC TCA -3') were used for the second round (Joag et al., 1996). For the first round of amplification, 10 μ l of DNA (containing 0.5–1 μ g) were added to 40 μ l of an amplification solution, or "mix1" containing: 5 μ l of reaction buffer (10X) (670 mM Tris/HCl (pH 8.8), 160 mM $(\text{NH}_4)_2\text{SO}_4$, 0.1% Tween-20), 5 μ l of MgCl_2 (50 mM), 1 μ l of dNTP (25 mM of each oligonucleotide triphosphate: dATP, dGTP, dCTP, dTTP), 0.25 μ l of TAQ polymerase (5 U/ μ l, Eurobiotaq[®] DNA Polymerase-Thermostable, GAETAQ02K, EUROBIO, Les Ulis, France), 0.5 μ l of each primer GAG EX3, GAG EX5, ES30 and ES32 (20 μ M, GIBCO BRL Custom primers – Life Technologies, Grand Island, NY) and 26.75 μ l of DEPC-treated water. For the second round, 5 μ l of the first round were added to 45 μ l of a second amplification solution, or "mix2" containing the same reagents as the solution in mixture 1, except that internal primers GAG IN5, GAG IN3, ES31, and ES33 were used in place of external primers GAGEX5, GAGEX3, ES30, and ES32. For each round, following initial denaturation at 94°C for 5 min, the samples were submitted to a series of 35 cycles comprising, successively, a further 1-min denaturation phase at 94°C, a 90-s hybridization phase at 46°C and a 2.5-min extension phase at 60°C. Each round was followed by a final extension at 60°C for 15 min.

Amplification products were visualized using electrophoresis on 1.5% agarose gel (Gibco Life Technologies, Grand Island, NY), containing ethidium bromide in 1X TAE buffer: 10 μ l of the amplified fraction were added to 5 μ l of dyed loading buffer, in each gel well. Two controls were performed for each gel: a positive control (CAEV proviral-DNA from infected GSM (goat synovial membrane)) and a negative control (distilled water). 5 μ l of smart-ladder (Gibco Life Technologies) was used as a molecular weight marker. This marker comprises 14 bands calibrated between 10,000 and 200 bp. Following electrophoretic separation, the bands were visualized using transillumination, with ultraviolet light (312 nm). The sensitivity of this technique enables the detection of one in vitro-infected GSM (goat synovial membrane) cell and a minimum of 10 in vitro-infected fibroblastic cells (Chebloune et al., 1996).

6. Statistical analysis:

The true prevalence of serologically positive animals was estimated by adjusting the apparent prevalence to the sensitivity and specificity of the test using the following equation (Rogan and Gladen, 1978):

$$TP = AP + SP - 1 / Se + Sp - 1$$

where TP is true prevalence, AP the apparent prevalence, Sp the test specificity, and Se is the test sensitivity.

The correlation between the presence of CAEV in the serum and in the blood was determined using the χ^2 -test with Yates' correction. A p-value of equal to or less than 0.05 was considered significant. SPSS software[®] was used for all statistical analysis.

Results

1. CAEV serological survey:

Forty seven goats out of the 243 surveyed from the nine farms and 47 goats also out of the 176 surveyed from abattoir were seropositive to CAEV. The true prevalence (TP) of seropositive individual animals, as adjust to the specificity and sensitivity of the ELISA test, was 15.7 % (Table 1).

Table 1. Results of ELISA and PCR of the blood samples of investigated local goats in Syria.

Results of PCR and ELISA		(n)	(%)
ELISA	Positive	47	19.34
	Negative	196	80.66
	Total	243	100
PCR	Positive	16	6.58
	Negative	227	93.42
	Total	243	100
PCR and ELISA	PCR ^{Positive} and ELISA ^{Positive}	12	4.94
	PCR ^{Positive} and ELISA ^{Negative}	4	1.65
	PCR ^{Negative} and ELISA ^{Positive}	35	14.40
	PCR ^{Negative} and ELISA ^{Negative}	192	79.01
	Total	243	100

2. Detection of CAEV proviral-DNA by PCR:

Samples analyzed for CAEV proviral-DNA using PCR were considered as being positive when a 512 bp band, corresponding to the positive control, was seen on agarose gel electrophoresis under UV light, between the 600 and 400 bp molecular weight bands. Whereas the 393 bp band, generated from the amplification of the endogenous actin gene, was present in both non-infected and CAEV-infected samples. An example of the results is displayed in Figure (1), showing the analysis of DNA isolated from blood cells and genital tract tissue from a female goat.

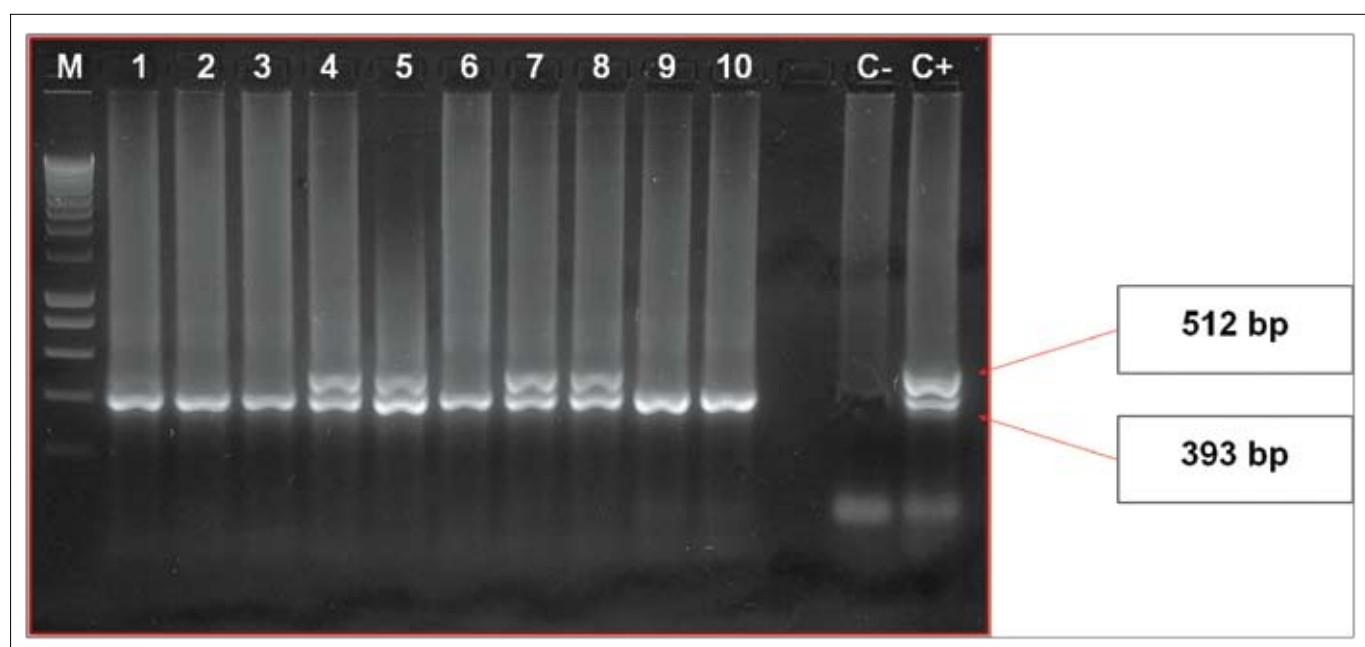


Fig 1. Example of nested-PCR amplification of proviral DNA. DNA from blood cells and genital tract tissues was used to perform nested-PCR using specific sets of oligonucleotide primers to amplify both the 512 bp CAEV gag and 393 bp-actin fragments. Following nested-PCR reactions, each PCR product was separated on 1.5% agarose gel and the bands visualized by staining with Ethidium Bromide. M = Smart Ladder used as a molecular weight standard. Lanes: 1, 2, 3, 6, 9 and 10, negative samples. Lanes: 4, 5, 7 and 8, positive samples. C+: positive control (CAEV proviral-DNA from GSM infected cells). C-: negative control (distilled water).

Nested-PCR amplification using DNA isolated from the circulating blood cells resulted in clear bands of the expected size (512 bp) on agar gel electrophoresis for 16 of the 243 goats were examined. The presence of CAEV proviral-DNA was significantly higher ($P < 0.001$) in seropositive goats (Table 1). The various genital tract tissue samples from 47 seropositive goats were analyzed using PCR. Seventeen samples (9 uterus, 4 ovaries and 4 oviducts) from 11 out of 47 goats, i.e. 23.40%, presented at least one PCR positive tissue sample (Table 2).

Table 2. Results of PCR tests on the genital tract tissue samples of 47 seropositive investigated local goats.

		Uterus n (%)	Ovaries n (%)	Oviducts n (%)
PCR	Positive	9 (19.15)	4 (8.5)	4 (8.5)
	Negative	38 (80.85)	43 (91.5)	43 (91.5)
	Total	47	47	47

Discussion

The main objective of our study was to determine the seroprevalence of caprine arthritis-encephalitis virus (CAEV) infection, and to investigate the infectious status of blood and genital tract tissues from goats naturally infected with CAEV in Syria. Samples taken from 243 Shami and local goats were examined for CAEV specific antibodies and proviral genome in blood. Samples taken from 176 goats from the slaughter-house were tested for CAEV specific antibodies and genital tract tissues (uterus, oviducts and ovaries) from 47 seropositive goats were examined for DNA-proviral of CAEV.

Out of 419 (243+176) sera, using commercially available ELISA test, 22.4% showed a positive reaction. This finding indicates that CAEV is endemic in Syria. This seroprevalence was higher than that reported in Turkey (1.9%), southern Mexico (3.6%), and Great Britain (10.3%) (Burgu *et al.*, 1994; Dawson and Wilesmith, 1985; Torres-Acosta *et al.*, 2003). On the other hand, the prevalence reported in this study was lower than that reported in Jordan (32.2%), Central Mexico (28.6%), Wales (56.8%), Australia (82%), and USA (73%) (Adams *et al.*, 1984; Al-Qudah *et al.*, 2006; Cutlip *et al.*, 1992; Greenwood *et al.*, 1995; Grewal *et al.*, 1986). Previous investigation in Syria, using agar immuno-diffusion test indicated a prevalence of 12.5%. However, this survey was conducted on a target population and only analyzed 72 samples (Giangaspero *et al.*, 1992).

Despite the detected seropositiveness, no clinical evidence of the infection was observed in the tested animals. This could be due to viral load, frequency of exposure, route of infection/re-infection, and the genetic makeup of the host and/or the local virus strain, since virulence and tissue tropism may vary with the strain (Thormar, 2005).

Small Ruminant Lentiviral Viruses (SRLV) heterogeneity and low viral load have hampered the use of the Polymerase Chain Reaction (PCR) as a reference technique to diagnose SRLV infection. Together with ELISAs based on peptides or recombinant antigens, double and/or nested PCR can improve specificity and sensitivity compared with using ELISA or simple PCR procedures alone (De Andres *et al.*, 2005; Extramiana *et al.*, 2002; Reina *et al.*, 2006). On the other hand, PCR has often been used to complete serological methods, since PCR positive results have been obtained in some seronegative animals (De Andres *et al.*, 2005; Gonzalez *et al.*, 2003). In our study, 4 goats from 16 PCR blood cell-positive goats were seronegatives. However, in nursing lambs from infected dams, ELISA positive results due to colostral antibodies can be obtained in PCR-negative samples (Alvarez *et al.*, 2005). So far, peripheral blood mononuclear cells are the main source of target DNA. Milk, colostrum, semen, synovial fluid and genital tract tissues give a lower sensitivity and are less reliable than blood as a SRLV DNA source for PCR (De Andres *et al.*, 2005;

Extramiana et *al.*, 2002). This explains the low tissue DNA recovery rate that was obtained in this study.

PCR testing on blood samples revealed that 6.6% of the goats used during this study were infected. This frequency was lower than that reported in industrialized countries (Cheevers et *al.*, 1988; East et *al.*, 1987). In fact, we demonstrated a positive correlation between seropositive goats and genital tract tissue infection ($P < 0.05$). Indeed, 23.4 % of the animals with positive tissue were also seropositive test. The absence of amplification of proviral-DNA in the genital tract of the other goats could be explained by an unequal distribution of the CAEV-infected cells in these tissues, and the small quantity of tissue sample taken for examination.

In conclusion, this investigation further shed lights on the importance of CAEV infection in goats in Syria, which in return should alarm the Ministry of Agriculture authorities to initiate control strategies for this rather economically important infectious disease.

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Ref : 345 / Accepted 9 - 2013