



Short communication

Diagnosis of caseous lymphadenitis by ELISA in naturally infected goats from Venezuela

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ARTICLE INFO

Article history:

Received 14 January 2009

Received in revised form

23 September 2009

Accepted 29 September 2009

Available online 29 October 2009

Keywords:

Corynebacterium pseudotuberculosis

Goat

ELISA

Caseous lymphadenitis

ABSTRACT

Due to the absence of previous reports, the goal of this work was to detect caseous lymphadenitis (CLA) in goat flocks from Venezuela using an indirect immunoenzymatic assay (ELISA). Eighteen farms were randomly selected in Falcon State, North-Western Venezuela. Blood samples were taken from 259 goats, 65 of them with abscesses. Experimental inoculations were made to healthy kids with 0.5 mL inocula containing 4.7×10^5 of *Corynebacterium pseudotuberculosis* to observe the kinetics of antibody response. Immunoenzymatic assays were carried out using exotoxin of *C. pseudotuberculosis* as antigen. Antibody response in experimentally inoculated animals was detected 2 weeks after infection. Of 259 field goat sera, 55.98% were positive by ELISA. Of 65 goats with abscesses, 67.69% had CLA demonstrated by bacteriological methods; from these, 72.73% showed antibodies by ELISA. Of the remaining goats negative to CLA, 47.62% had antibodies by ELISA. Sensitivity was calculated in 72.73% and specificity in 67.74%. The immunoenzymatic assay applied in this research could be useful to detect CLA in naturally infected goat flocks from Venezuela.

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1. Introduction

Several serodiagnostic tests have been developed worldwide to overcome the problem of clinical identification of caseous lymphadenitis (CLA) in small ruminants with variable results (Derksen et al., 2000; Dorella et al., 2006; Seyffert et al., 2009). Immunoenzymatic methods are invaluable tools to diagnose the disease in those regions where molecular methods are not available as in Venezuela. In this work, an indirect ELISA with *Corynebacterium pseudotuberculosis* autochthonous strains was used to detect CLA in Venezuelan goat flocks.

2. Materials and methods

2.1. Sampling

Miranda County (Falcon State, North-Western Venezuela) has a goat population of 67,528 heads, distributed in 423 farms (Secretaría de Desarrollo Agrícola, 2006). From these, 18 farms with a total of 2604 animals were selected at random. Blood samples for serological tests were collected of 194 animals of both sexes and several ages or breeds with no signs of the disease, and 65 goats with clinical CLA lesions, from which pus samples were also collected and tested by bacteriological methods. Data concerning animals (age, breed, and sex) were also recorded.

2.2. Bacteriological characterization of *C. pseudotuberculosis*

Each pus sample from field and experimental animals was processed as in Chirino-Zárraga et al. (2006).

2.3. Strains of *C. pseudotuberculosis*

Strain C32B was isolated from a Nubian male goat, 3 years old, which present two closed abscesses in superficial cervical lymph nodes; refer-

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ence strain 19410 of *C. pseudotuberculosis* was acquired from American Type Culture Collection (ATCC), Atlanta, USA. C32B was grown in 5% bovine blood agar (Himedia, Mumbai, India) with a modification of *ter Laak et al. (1992)*; after 48 h in 37 °C and 5% CO₂ (*Jones and Collins, 1986*), one colony was transferred to 5 mL nutrient broth (Himedia, Bombay, India) supplemented with 0.1% Tween 80 for 24 h. From this, 1 mL was inoculated in 50 mL nutrient broth–Tween 80 for 24 h more without agitation. The bacteria were successively diluted in sterile saline solution (0.85% NaCl) to obtain colony forming units (CFU). Reference strain was cultured as directed by ATCC.

2.4. Preparation of antigen for the ELISA

Three colonies from each strain, previously incubated as above, were grown in 1 L of 0.1% Tween 80–BHI broth (BBL, Le Pont de Claix, France) for 72 h and then overnight at 4 °C. The culture was centrifuged at 13,800 × g for 30 min at 10 °C. Supernatants were collected and thymerosal was added to a final concentration of 1:10,000. The protein concentration of both C32B and ATCC 19410 supernatants (12.50 and 12.64 µg/µL, respectively) was estimated as in *Lowry et al. (1951)*. Supernatants containing the exotoxin were stored in aliquots of 2 mL at –20 °C for further use. *C. pseudotuberculosis* complete bacteria to be used as antigen in ELISA were obtained as explained in Section 2.3.

2.5. Experimental inoculation

Four Nubian kids of 3–5 months old, born in a flock free of *C. pseudotuberculosis*, clinically healthy, were maintained in isolation facilities supplied with water, hay and grass *ad libitum*. Two of them (1 and 2) were inoculated with 0.5 mL of *C. pseudotuberculosis* (strain C32B) suspension containing 4.7×10^5 CFU by subcutaneous route in the right escapular region (week 0). Fifty-six days later (week 8) a second dose of 0.5 mL containing 5.3×10^8 CFU was injected subcutaneously in the right abdominal region of both animals as in *ter Laak et al. (1992)*. Kids 3 and 4 were inoculated subcutaneously with 0.5 mL of sterile saline solution in the right escapular region and were maintained as negative controls. Sera were taken on 41, 21 and 0 days before inoculation, and weekly until day 105 (week 15) after inoculation with *C. pseudotuberculosis*. Samples of the abscesses were taken from kid C1 at weeks 5 and 9. Bacteriological characterization was made as in *Chirino-Zárraga et al. (2006)*.

2.6. ELISA

To standardize the conditions of ELISA, several concentrations of antigen (exotoxin and *C. pseudotuberculosis* suspensions) were tested. Sera from goats with CLA were used as positive controls. Sera from 1 to 5 weeks prior to inoculation were used as negative controls. Polystyrene plates (Cliniplate EB, Labsystem, USA) were coated with increasing concentrations of C32B or ATCC 19410 exotoxin or *C. pseudotuberculosis* suspension (6.25, 10, 20 and 40 µg/mL) diluted in bicarbonate–carbonate buffer, pH 9.6 and incubated overnight at 4 °C. Wells were washed three times with 200 µL phosphate saline buffer (PBS) containing 0.1% Tween 20. Non-specific binding sites were blocked with 0.5% gelatin for 1 h at 37 °C. Positive and negative sera diluted 1:100 in PBS–0.1% Tween were added in duplicate. Following incubation for 1 h at 37 °C, and washed again, wells were filled with 100 µL of horseradish peroxidase anti-goat immunoglobulin conjugate (Sigma® cat. A-5420, USA) diluted 1:30,000 in PBS–Tween buffer. Optimal dilution of horseradish peroxidase anti-goat immunoglobulin conjugate was previously obtained against goat IgG (Sigma® cat. I-9140, USA). After incubation of 1 h at 37 °C, conjugate solution was discarded and washed as before. Each well was then filled with 100 µL of ABTS (2,2'-azino-di-(3-ethyl-benzothiazole-sulphone-6)-dianiline) (Sigma® cat. A-1888, USA) in appropriate buffer. The plates were left at room temperature in darkness for 3 h on a shaker. Absorbance values were obtained and recorded by an ELISA plate reader (Awareness, Stat Fax 2100, USA) at 405 nm. Once optimal concentration of antigen was achieved, conditions for running test with field sampled sera were the same as explained above. Evaluation of experimental sera was made with *C. pseudotuberculosis* suspensions as antigen. The best positive/negative sera ratio was obtained with the strain C32B exotoxin concentration of 10 µg/mL so it was used for screening field samples.

2.7. Estimation of the cut off point

Ten sera from health experimental animals were used as negative controls. The cut off point was established as in *Sheldrake et al. (1983)*. Using exotoxin, the cut off point of OD₄₀₅ = 0.1475. With *C. pseudotuberculosis* suspensions as antigen and negative control sera, cut off point of OD₄₀₅ = 0.427. Five sera were used as positive controls (three experimental from weeks 10, 11 and 14 post-infection and two sera of naturally infected goats, from which *C. pseudotuberculosis* had been previously isolated). Specificity and sensitivity were calculated as per *Tarabla (2000)*.

2.8. Statistical analysis

Chi-square test was used for analysis of age and breed differences and for comparison between ELISA and bacteriological results (*Snedecor and Cochran, 1967*). The level of significance applied to data was $p < 0.05$.

2.9. Interpretation of results

The criteria taken for considering positive or negative animals to CLA were as follows:

Bacteriological isolation	ELISA	Interpretation
+	+	Positive
+	–	Positive
–	+	Doubtful
–	–	Negative

3. Results

3.1. Pathological observations through necropsy in experimentally inoculated animals

An important inflammatory response was observed through the first week post-inoculation; at second week, acute symptoms developed toward a chronic suppurative state in kid 1; in kid 2, at fifth week. One abscess in scapular subcutaneous tissue was found in kid 1. Overgrown right superficial cervical and subiliac lymph nodes (4 cm × 4 cm), with seven piogranulomatous processes around were found in the kid 2. Three abscesses were also observed in inoculation site. *C. pseudotuberculosis* was demonstrated in cultures of the overgrown lymph nodes and the abscesses found in inoculation site in both kids.

3.2. Selection of exotoxin as antigen in ELISA

Exotoxin isolated from field *C. pseudotuberculosis* isolates was chosen due to a best positive:negative sera ratio (≥ 4) compared with those of ATCC exotoxin. ELISA with *C. pseudotuberculosis* antigen gave a lower positive:negative sera ratio (≤ 2).

3.3. Development of antibodies in experimentally inoculated animals

Antibody response against *C. pseudotuberculosis* antigen was detected at 2 weeks post-inoculation in kids 1 and 2. Average values of absorbance in both kid sera ranked between 0.250 ± 0.064 and 0.595 ± 0.07 along the experimental trial. On week 9, one week after reinoculation, antibody response elevated in both animals and maintained until the end of the trial. Uninoculated animals (kids 3 and 4) did not develop antibody response against *C. pseudotuberculosis* antigen (Fig. 1).

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