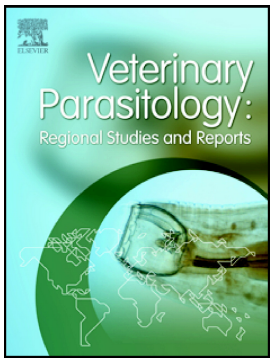


Seropositivity to Sarcocystis infection of llamas correlates with breeding practices

Sandra Romero, Tamara Carletti, Cecilia Decker Franco, Gastón Moré, Leonhard Schnittger, Monica Florin-Christensen

PII: S2405-9390(16)30242-8
DOI: doi: 10.1016/j.vprsr.2017.08.006
Reference: VPRSR 124

To appear in: *Veterinary Parasitology: Regional Studies and Reports*

Received date: 17 October 2016
Revised date: 15 August 2017
Accepted date: 15 August 2017

Please cite this article as: Sandra Romero, Tamara Carletti, Cecilia Decker Franco, Gastón Moré, Leonhard Schnittger, Monica Florin-Christensen , Seropositivity to Sarcocystis infection of llamas correlates with breeding practices. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Vprsr(2017), doi: 10.1016/j.vprsr.2017.08.006

Seropositivity to *Sarcocystis* infection of llamas correlates with breeding practices

Sandra Romero[1], Tamara Carletti[2], Cecilia Decker Franco[2], Gastón Moré[3,4], Leonhard Schnittger[2,4], Monica Florin-Christensen[2,4,*]

[1]Instituto de Investigación y Desarrollo Tecnológico para la Agricultura Familiar Región NOA, Instituto Nacional de Tecnología Agropecuaria (INTA), RN 9 Km 1763, (4622) Posta de Hornillos, Argentina

[2]Instituto de Patobiología, Centro de Investigaciones en Ciencias Veterinarias y Agronómicas (CICVyA), INTA, Los Reseros y Nicolas Repetto, s/n, (1686) Hurlingham, Argentina

[3]Laboratorio de Inmunoparasitología, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, 60 y 118, (1900) La Plata, Argentina

[4]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), (C1425FQB) Buenos Aires, Argentina

*Corresponding author:

Phone/FAX: +541146211289
e-mail: jacobsen.monica@inta.gob.ar

Abbreviations: SAC: South American camelid

Abstract

Production of llama (*Lama glama*) meat in rural communities of the Andean regions is largely affected by *Sarcocystis* spp. infection. Macroscopic cysts develop in muscles as a consequence of *S. aucheniae* parasitism, often resulting in meat downgrade or condemnation. Llama meat production is informal in Argentina but has broad perspectives for improvement, and would significantly benefit from the development of standardized control methodologies. This work analyzes whether the presence of anti-*Sarcocystis* spp. antibodies in llamas is influenced by factors such as geographic region and/or herd management practices. To this aim, an indirect ELISA was set up based on a ~23 kDa soluble immunogenic protein fraction (Sa23), isolated from *S. aucheniae* macrocysts (Sa23-iELISA). Serum samples (n=507) were collected from llamas bred under three different conditions: (i) with no sanitation controls and in the presence of pastoral dogs by small producers of different localities of the Argentine Puna (Group I, n=237); (ii) with sanitation controls and no pastoral dogs, in fenced fields of an experimental agricultural station in the Argentine Puna (Group II, n=167); and (iii) with sanitation controls and no pastoral dogs in fenced fields of farms of the humid Pampas (Group III, n=103). Results of the Sa23-iELISA were expressed as percentages of positivity with respect to a reference *Sarcocystis*-positive serum. Notably, the percentage of sera that fell above the cut-off (31.5% positivity) in group (i) was significantly higher ($p < 0.001$) than those of groups (ii) and (iii) (50% *vs* 23% and 26%, respectively). These results indicate that herd management practices constitute a critical risk factor for sarcocystiosis in llamas. Differences in these practices include feeding of dogs with raw *Sarcocystis*-infected llama meat, with the consequent maintenance of the parasite life cycle by the contamination of pastures and water with fecal-derived infective oocysts/sporocysts. Additionally, the itinerancy of llama herds in

search for pastures and water sources possibly exposes animals to a higher number of infective foci. On the other hand, percentages of seropositive llamas kept under controlled conditions in the Puna or the humid Pampas were not significantly different, suggesting that climate, altitude, and/or pasture characteristics do not influence *Sarcocystis*-infection. Male gender and older age of llamas were found to be propensity factors for sarcocystiosis in llamas bred in La Puna under controlled conditions. Availability of diagnostic tools, as well as increased knowledge on the parasite and its epidemiology, will allow the design of control strategies for SAC sarcocystiosis.

Keywords

Llama; sarcocystiosis; *Sarcocystis aucheniae*; serology; breeding

1. Introduction

Domestic South American camelids (SACs; llamas and alpacas) are bred in Argentina, Chile, Bolivia, Peru, and Ecuador, adding up to a population of about 7 million animals (FAO 2005a, b; Lamo, 2011). SAC breeding was extensive and widespread in pre-hispanic South America, but after European colonization, it became increasingly restricted to the High Plateau area (Puna), where non-indigenous productive cattle cannot thrive (Dufour et al., 2014). Nowadays, the highest proportion of SACs is raised in small herds by Andean rural families, with pastoralism practices and no sanitary controls (Markemann and Valle Zárate, 2010). However, medium and large-scale SAC breeders also exist in Andean and non-Andean regions. SACs are a source of meat, fine fiber, and dung used as fertilizer and combustible, and are also deployed for transport and company (Leguía, 1991). Indeed, SAC meat is commonly consumed in Andean communities, yet is becoming increasingly used for gourmet cuisine and by health-oriented consumers, due to its good taste and high protein/cholesterol ratio (Mamani-Linares and Gallo, 2014). Importantly, SACs have a lower ecological impact than cattle regarding water consumption, earth erosion, and carbon emissions (Saadoun and Cabrera, 2008; Wheeler, 2012; Dittman et al., 2014). Thus, SAC meat potentially is a highly valuable good for local and international markets.

A factor that severely affects SAC meat production is the infection with the Apicomplexan protozoon *Sarcocystis aucheniae* that produces macroscopic cysts (1 to 5 mm-long) in skeletal muscle (Carletti et al., 2013; Rooney et al., 2014; Moré et al., 2016). Recently, an additional species, *S. masoni*, has been described producing microscopical cysts in SACs from Argentina and Perú (Moré et al., 2016). Infections with both *Sarcocystis* spp. are usually asymptomatic, although cases of eosinophilic myositis and abortion associated to acute sarcocystiosis (also known as "sarcocystosis") have been reported (La Perle et al.,

1999; Gabor et al., 2010). Human consumption of undercooked *S. aucheniae*-infected meat leads to gastroenteritis, chills, nausea, diarrhea, colic, and respiratory problems (Leguía, 1991). However, subjecting meat to high temperatures or drying inactivates the toxin that causes these symptoms (Godoy et al., 2007). Indeed, the main negative effect of this parasitosis is the repulsive aspect that macrocysts confer to meat, preventing its commercialization or significantly lowering its price, with important economic losses for the producers (Leguía and Casas, 1999; Rooney et al., 2014). *S. aucheniae* macroscopic cysts have not only been found in llamas and alpacas but also in the guanaco (*Lama guanicoe)*, a wild SAC distributed in the Southern regions of South America (Regensburger et al., 2015).

Dogs, considered to be the main definitive hosts of *S. aucheniae*, eliminate abundant infective sporocysts with the feces, after being fed raw infected meat (Choque et al., 2007). In the case of *S. masoni*, the definitive host is still unknown, although canids have been suggested to fulfill this role (Moré et al., 2016). SACs acquire sporocysts when taking up infected pastures and/or water. After reaching the intestine, parasites undergo invasion, stage transformation and multiplication events until they reach the muscles, where they invade myocytes and encyst (Dubey et al., 1989). They then multiply to high densities by merogony, reaching an average of 20 million bradyzoites per *S. aucheniae* cyst (Carletti et al., 2013). It is possible to detect *S. aucheniae* during its passage through the SAC circulatory system by molecular methods (Martin et al., 2016). Also, parasites stimulate the host immune system leading to a humoral response (Moré et al., 2008).

Few reports on the epidemiology of SAC sarcocystiosis are so far available. A study carried out in Bolivia abbatoirs estimated that 34.1% llama carcasses examined in the period between 2006 and 2011 presented macroscopic cysts (Rooney et al., 2014). In addition, an

immunofluorescence assay (IFAT) using *S. aucheniae* bradyzoites and an indirect ELISA based on soluble *S. aucheniae* protein extracts were developed for the serological diagnosis of SAC sarcocystiosis (Castro et al., 2004; Medrano et al., 2006; More et al., 2008). However, neither of these methods has been validated. Percentages of seropositive SACs as determined by IFAT or iELISA were around 90% for llamas in Jujuy, Argentina, and alpacas in Peru; although these high values were likely influenced by a high degree of cross-reactivity at the genus level (Castro et al., 2014; Medrano et al., 2006; More et al., 2008).

In this study, we hypothesized that SAC breeding conditions and/or geographical distribution influence *Sarcocystis* infection as measured by the detection of *Sarcocystis*-specific antibodies. To test this hypothesis, we developed an iELISA based on an immunogenic protein fraction of *S. aucheniae*, to increase the reliability of the serological determinations with respect to previously developed methods. This iELISA was applied to samples of llamas (i) originating from two different geographic regions: Puna and the humid Pampas, and (ii) bred under two different management practices: in fenced areas with sanitation controls, and by itinerant shepherds, in the presence of pastoral dogs and with scarce sanitation controls.

2. Materials and Methods

2.1. Collection of llama serum samples

A total of 507 llamas from Argentina were sampled. An aliquot of 5 ml blood was aseptically collected from the jugular vein of each animal. The head of the animals was previously covered with a cloth hood to avoid stress, as recommended by Marcoppido and Vila (2013). Serum was separated and stored at -20 ^{o}C until use. The samples were divided

according to their origin and/or llama breeding conditions, as follows. Group I (n= 237): llamas bred in small mixt herds by rural families in the Puna localities of Yavi, Santa Catalina, Cochinoca, Susques, and Rinconada, Province of Jujuy. The total number of llamas in this region was estimated at approximately 137,500 (Echenique et al., 2015). Group II (n= 167): llamas bred in the experimental station of the National Institute of Agricultural Technology (INTA) in the Puna locality of Abra Pampa, Jujuy, from a total of 640 animals; and Group III (n= 103): llamas bred in the humid Pampas, in private farms of the Province of Buenos Aires and Entre Rios, and at the experimental field of the National Institute of Agricultural Technology (INTA) at Castelar, Buenos Aires. The total number of animals in the humid Pampas is estimated at approximately 12,000 (Carlos Poplavsky, personal communication). While the Puna is characterized by its high altitude, aridity, large thermal amplitude, and poor pastures, the provinces of Entre Rios and Buenos Aires are at sea-level, have humid, temperate weather, and rich pastures. Llamas from groups II and III lived in fenced areas, in the absence of dogs, and had regular sanitary controls, including veterinary check-ups, vaccinations, disease diagnosis and treatment if needed. In contrast, llamas from Group I were bred under informal conditions, with no sanitary controls, in the presence of pastoral dogs, and were regularly moved through different fields in the search for pastures. Pastoral dogs of this group were regularly fed with uncooked llama meat. Sampled animals were from both sexes, older than 4 months, and their origin and management conditions were recorded. Llamas from Groups I and III were bred in herds of 50 to 130 animals.

2.2. Determination of *Sarcocystis*-positive and negative llama sera by immunofluorescence (IFAT)

IFAT was carried out as described by Moré et al. (2007). Briefly, *S. aucheniae* macrocysts were separated from a sample of llama meat from Puna, Argentina, and excised with a scalpel. Free bradyzoites contained in the cyst were collected, quantified by microscopic counting in a Neubauer hemocytometer and diluted with PBS to a density of $4x10^6$ parasites/ml. Aliquots of 10 µl were deposited in each well of immunofluorescence glass slides and allowed to dry. Wells were incubated with test and reference sera (diluted 1:50 in PBS) for 30 min at 37°C, washed and incubated with fluorescein isothiocianate (FITC)-conjugated goat anti-bovine IgG (Sigma) for further 30 min, followed by washing. Cover slides were mounted with glycerol/PBS, and slides were observed in an epifluorescence microscope with 400 x magnification.

2.3. Identification, characterization, and purification of a *S. aucheniae* antigenic fraction

Suspensions of bradyzoites, collected from macroscopic *S. aucheniae* cysts and suspended in PBS, were homogeneized either with a Branson sonifier (5 pulses of 1 min each, setting 5) or a FastPrep-24 equipment (3 pulses of 25 s each). Phenylmehtylsulfonide fluoride (PMSF) was added to a final concentration of 1 mM, and protein concentration was determined using a Micro BCA ProteinAssay Kit (Pierce). Proteins (350 µg) were separated by preparative SDS-PAGE in 12.5% polyacrylamide minigels, using PageRuler Prestained Protein Ladder (ThermoScientific) run in a separate lane as marker, followed by electro-transference to a nitrocellulose membrane (Whatman). After overnight incubation at 4 °C with blocking buffer (3% skimmed milk/0.1% Tween-20/PBS), the membrane was cut in 3 mm-wide stripes. Different stripes were incubated (1 h, 37 °C) with llama sera (n=10) diluted 1:20 in blocking buffer. Five of these sera were IFAT-positive and five were IFAT-

negative against *S. aucheniae* bradyzoites at dilutions equal to or higher than 1:50, and corresponded to llamas from Jujuy and Buenos Aires provinces, respectively. After three 10 min washes with PBS/0.1% Tween-20, stripes were incubated (1 h, 37 ^{o}C) with horse radish peroxidase-conjugated goat anti-llama IgG (Bethyl Laboratories Inc.), diluted 1:1500 in blocking buffer, followed by three washes as before. Reactions were visualized by incubation with DAB substrate (0.62 mg/ml 3,3′-diaminobenzidine tetrahydrochloride/ 20 mM Tris-HCl, pH 7.4/ 0.15 M NaCl/0.003% (v/v) hydrogen peroxide) until the appearance of brown color and then stopped by dilution with distilled water. A band of 22.8 kDa (here referred to as Sa23), as determined by extrapolation from a plot of log molecular weight *vs* relative mobility (Rf) of protein standards, was selected as immunodominant antigen for the iELISA.

To analyze the subcellular localization of Sa23, a *S. aucheniae* bradyzoite lysate (200 µl, 1 mg protein/ml), obtained by FastPrep-24, was subjected to Triton X-114 partition to obtain total, soluble, and membrane-bound protein fractions, following the protocol of Wang and Coppel (2001). After separation, proteins were concentrated with ice cold 80% acetone (v/v, final concentration) overnight at -20°C, followed by centrifugation (5,000 *g*, 30 min, 4° C). Precipitated proteins were suspended in PBS, separated by SDS-PAGE in 15 % polyacrylamide minigels and analyzed by Coomassie blue and Western blot, using the *Sarcocystis*-positive and negative llama sera mentioned above.

Partially purified Sa23 was obtained by electro-elution and used as iELISA antigen. To this end, an aliquot of a bradyzoite homogenate (350 µg protein) was run by preparative SDS-PAGE and the gel portion containing proteins of ~23 kDa was excised and transferred to a dialysis bag containing 2 ml PBS. The dialysis bag was immersed in a horizontal

electrophoresis tray containing 25 mM Tris-HCl/ 250 mM glycine/ 0.1 % SDS, pH 8.8, and protein electro-elution was exerted applying an electric current of 35 mA for 150 min. The bag was then removed and dialyzed overnight against PBS at 4 ^{o}C. The content was recovered, aliquoted, and stored at -20 ^{o}C, after addition of 1 mM PMSF (final concentration). Protein concentration was colorimetrically determined as described above.

2.4. Set-up of an indirect ELISA (iELISA) based on Sa23 for the detection of *Sarcocystis*-specific antibodies

Optimal antigen concentrations and serum dilutions to be used in iELISA were determined in a pilot experiment. To this end, triplicate wells of two Immulon 2HB 96-well flat-bottom plates were incubated overnight at 4 ^{o}C with 50 µl of 1:2 serial dilutions of Sa23 (from 16 to 0.5 µg/ml) in carbonate/bicarbonate buffer (0.015 M sodium carbonate/ 0.035 M sodium bicarbonate, pH 9.6). Plates were washed thrice with PBS-0.2% Tween-20 (PBS-T) and incubated for 30 min at RT with 100 µl blocking buffer, which, for these set-up experiments, consisted of 0.5% bovine serum albumin in PBS-0.2% Tween-20 (PBS-T). After discarding the blocking buffer, 100 µl aliquots of 1:400, 1: 800 and 1:1600 dilutions in blocking buffer of either a *Sarcocystis*-positive or a *Sarcocystis*-negative llama serum (according to IFAT) were added to the wells, followed by incubation for 30 min (RT). After three washes with PBS-T, wells were incubated with 100 µl horse radish peroxidase-conjugated goat anti-llama IgG (1/1500 dilution) for 30 min (RT). Wells were then washed five times with PBS-T, after which 100 µl of TMB (3,3',5,5'–tetramethylbenzidine) chromogenic substrate were added, prepared by following the manufacturer´s guidelines (Sigma). After 15 min at RT, reactions were stopped with 100 µl 85 % (v/v)

orthophosphoric acid in H_20, and absorbance was recorded spectrophotometrically at 450 nm. A_{450} ratios between positive and negative sera were recorded for each condition and were maximal using 4 μg/ml antigen (0.2 μg/well) and a 1:800 serum dilution. Using these concentrations, the performance of other blocking reagents, namely 5 % v/v normal equine serum, 1% w/v ovalbumin, 0.5% w/v gelatin and skimmed milk (10, 15 and 20 % w/v), in all cases dissolved in PBS-T, was then examined. It was observed that 20 % skimmed milk in PBS-T yielded the highest positive/negative A_{450} ratios, and was thus chosen as blocking buffer. Control IFAT-negative and positive sera were included in each plate as controls. The A_{450} value of the negative control was subtracted from each determination, and percentages of positivity were calculated as the ratios between A_{450} of test samples and A_{450} of the reference positive serum, multiplied by 100. The test cut-off was determined as the average of the percentages of positivity of 36 IFAT-negative llama sera, plus 2 standard deviations.

2.5. Statistical analysis

The significance of the differences in the numbers of serologically positive llamas according to breeding practices/geographical location, and, in the case of Group II, according to age (2 years old or older and younger than 2 year), and gender, was evaluated by the Chi square test, using the statistical software STATA (StataCorp, 2015).

3. Results and Discussion

3.1. Identification and characterization of an immunogenic fraction in *Sarcocystis aucheniae* protein extracts

S. aucheniae bradyzoite proteins were tested by immunoblotting for immune reactivity against five *Sarcocystis*-positive and five *Sarcocystis*-negative llama sera, as determined by IFAT. Among several reactive bands, the positive but not the negative sera clearly recognized a 22.8 kDa protein fraction. The immunoblot corresponding to one of the tested sera is shown in Fig. 1. These results suggest that one or more proteins contained in this fraction, designated Sa23, constitute immunodominant antigens suitable for the development of serological tests.

To analyze whether the immunoreactive protein/s in Sa23 is soluble or membrane-bound, a *S. aucheniae* bradyzoite homogenate was subjected to Triton X-114 phase partition and aliquots of total proteins, and aqueous and membrane fractions were analyzed by immunoblot using *Sarcocystis*-positive and negative sera (Fig. 1). The ~23 kDa immunoreactive band was observed in the total protein and aqueous fractions, but not in the membrane-bound fraction, revealing its soluble nature. These results are indicative that Sa23 is not related to SAGs, a family of membrane-bound surface proteins partially conserved among coccidians, some of which have been used as antigens for the development of serological tests (Howe et al., 2005; Dangoudoubiyam et al., 2011).

3.2. Development of the Sa23 iELISA

Sa23 was partially purified from *S. aucheniae* total protein extracts by SDS-PAGE followed by electro-elution, and used as antigen for the development of an iELISA (Sa23-

iELISA). Antigen amounts, serum dilutions, and blocking reagent were chosen so that maximal A_{450} ratios between control IFAT-positive and negative sera were achieved. Briefly, the final Sa23-iELISA protocol consisted of the following steps: (i) sensitization of ELISA plates with the Sa23 antigenic fraction (0.2 μg/well), (ii) blocking with 20% skimmed milk in PBS-T, (iii) incubation with test and control sera in a 1:800 dilution, (iv) incubation with peroxidase-conjugated anti-llama IgG secondary antibody at a 1/1500 dilution, (v) reaction with TMB colorimetric substrate, (vi) stopping reactions after 15 min with orthophosphoric acid, and (vii) A_{450} determinations. Steps (ii), (iii), and (iv) were done at RT and lasted for 15 min. Three and 5 washes with PBS-T were carried out after steps (iii) and (iv), respectively. A positive and a negative control were included in each plate, and consisted of an IFAT-positive serum and PBS, respectively. The A_{450} value of the negative control was subtracted from each sample and percentages of positivity were then calculated as the A_{450} ratios between test samples and the positive control. The cut-off was established at 31.5% which corresponds to the mean percentage of positivity obtained for 36 IFAT-negative sera plus two standard deviations.

While discrimination between IFAT-positive and negative samples was very clear using Sa23-iELISA (see Section 2.3), homogenates of whole *S. aucheniae* cysts or purified bradyzoites as antigens yielded very low differences in absorbance between positive and negative samples (results not shown). Thus, in the present study, the Sa23-iELISA was applied as the method of choice to detect anti-*Sarcocystis* spp. antibodies in different llama herds. Given that there is no gold standard or validated technique for the serological diagnosis of SAC sarcocystiosis, the assessment of the sensitivity and specificity of the Sa23-iELISA could not be performed so far.

One additional species of *Sarcocystis* has been reported to infect the myocardium of SACs, producing microscopic cysts (Leguía et al., 1989, Moré et al., 2016). This species, initially referred to as *S. lamacanis* has been recently re-named *S. masoni* (Moré et al., 2016). Antigenic cross-reactivity between *S. aucheniae* and *S. lamacanis* has been previously indicated (Medrano et al., 2006). In addition, cross-reactivity among *Sarcocystis* species was shown by Moré et al. (2008), who detected llama anti-*Sarcocystis* antibodies by IFAT, using bradyzoites of either *S. aucheniae* or the bovine-infecting *Sarcocystis* species, *S. cruzi*. Thus, until more information is available, reactivity of anti-*S. masoni* antibodies against Sa23 cannot be excluded.. On the other hand, infection of llamas by the coccidians *Neospora caninum* and *Toxoplasma gondii* eliciting humoral responses has also been reported for herds of the province of Jujuy, Argentina (Moré et al., 2008). However, the percentages of IFAT-positive llamas in the latter study were only 4.6 and 30%, for *N. caninum* and *T. gondii* respectively, while the percentages of *Sarcocystis* spp. IFAT-positive animals were 96% or 77%, depending if *S. aucheniae* or *S. cruzi* bradyzoites were used as antigen. The observed large differences in positive testing animals strongly suggest a very low or even absent cross-reactivity between these coccidians.

3.3. Detection of anti-Sa23 antibodies in llamas from Argentina bred under different conditions

Sa23-iELISA was applied to 507 serum samples of llamas from Argentina. In total, 182 samples (35.9%) scored positive, i.e. their positivity percentages were above the cut-off threshold.

Samples were divided in three groups (Groups I, II and III) according to the breeding conditions of the sampled llamas, as described in Section 2.1., and percentages of positive

samples by Sa23-iELISA were calculated for each group. Notably, Group I showed a significantly higher ($p<0.001$) percentage of seropositive animals (49.8%) than Group II (22.8%) or Group III (26.2%). Since llamas of Groups I and II experienced similar conditions with respect to altitude, climate and pastures, our results suggest that there are other propensity factors that favor the incidence of SAC sarcocystiosis in Group I. Lack of good management practices in llama breeding, including the presence of pastoral dogs among the herds that feed from uncooked llama meat and spread oocysts/sporocysts in pastures and water with their feces is a likely explanation for these results (Choque et al., 2007). Such a dissemination effect of dogs for another feces-transmitted parasite, *Echinococcus granulosus*, in the Puna, Argentina, has been recently reported (de Costas et al., 2014). In addition, a possible dissemination by wild canids that might act as definitive hosts of SAC *Sarcocystis* spp., cannot be excluded. Furthermore, the itinerant grazing habits of llama breeders of Group I might increase the risk of exposure of llamas to feces of infected dogs (Markemann and Valle Zárate, 2010). Llamas of Groups II and III, on the other hand, were raised in fenced fields with similar sanitation controls and in the absence of pastoral dogs, but experienced a strong diversity in climate, altitude and/or pastures. In accordance with the assumptions made above, percentages of serologically positive animals were not significantly different between these two groups, suggesting again that parameters associated to geographic location and pasture quality are not risk factors for SA sarcocystiosis.

Importantly, in the case of Groups II and III, percentages of seropositive animals, although lower than in Group I, are not negligible. This indicates that the spread of *Sarcocystis*-infections among SAC herds could take place even in the absence of pastoral dogs, and underscores that there are aspects of the parasite life cycle that need further elucidation.

3.4. Distribution of *Sarcocystis* sp. seropositive llamas according to age and gender

Gender and age as propensity factors were analyzed exclusively in llamas of Group II, since the breeding conditions were the same for all animals in this group, and other variables could be ruled out. The percentage of seropositive animals in the age group of 2 years or older (n=91; 28.5% positive) was 5.6 times higher ($p<0.001$) than that of the group younger than 2 years of age (n=59; 5.1% positive). A similar connection between age and *S. aucheniae* infection was observed by Castro et al. (2004) and Rooney et al. (2014), as analyzed by detection of macroscopic cysts at the time of slaughter or seroprevalence, respectively. The higher percentage of positivity in older animals could be connected to an accumulative exposure to infective forms of the parasite with increasing age. A similar correlation between host age and risk of infection was observed in the case of *Sarcocystis* spp. infecting dromedary camels (Hamidinejat et al., 2013).

When results were analyzed according to gender, Group II showed an almost 4 times increased ($p<0.001$) percentage of seropositive males (n=63, 33.3% positive) as compared to seropositive females (n=95, 8.4% positive). Since there were no differences in the breeding of males and females in this group, the most plausible explanation for this difference could be a negative effect of male hormones on the susceptibility to acquire this type of infection. In contrast, Rooney et al. (2014) reported that the finding of cysts at the time of slaughter was higher in females than in males in llamas of different regions of Bolivia. More studies are needed to analyze the significance of these differences.

4. Conclusion

This study presents a new iELISA for the detection of anti-*Sarcocystis* antibodies in SACs based on a ~ 23 kDa immunogenic protein fraction extracted from *S. aucheniae* bradyzoites. Since clinical signs in the intermediary host are rare and finding of *Sarcocystis* infection is normally done post mortem, such a serological test is a useful tool for epidemiological studies. The application of this Sa23-iELISA to samples of llamas of different origins showed a direct connection between the type of breeding and the presence of anti-*Sarcocystis* spp. antibodies. This information needs to be taken into account at the moment of implementing control measures and education campaigns aimed at minimizing the negative impact of SAC sarcocystiosis. Further research will be carried out to identify the protein/s responsible for the immunogenicity of the Sa23 fraction and also, to evaluate whether correlations exist between serological, molecular and histopathological detection of *S. aucheniae* infections in SACs.

5. Acknowledgments

This work was supported by the National Research Council (CONICET, PIP 114-200801-00653), the National Institute of Agricultural Technology (INTA, SALJU320051 and PRET SALJU1232204) and the Ministry of Science and Technology (MINCyT, PICT 2014-3747), Argentina. Provision of llama serum samples from INTA-Castelar by Dr. Gisela Marcoppido is gratefully acknowledged.

6. Bibliography

Carletti, T., Martin, M., Romero, S., Morrison, D.A., Marcoppido, G., Florin-Christensen, M., Schnittger, L., 2013. Molecular identification of *Sarcocystis aucheniae* as the macrocyst-forming parasite of llamas. Vet. Parasitol. 198, 396-400.

Castro, E.C., Sam, R.T., Lopez, T., Gonzalez, A., Silva, M., 2004. Evaluación de la edad como factor de riesgo de seropositividad a *Sarcocystis* sp. en alpacas. Rev. Inv. Vet. Peru 15, 83-86.

Choque, J., Chavez, A., Pacheco, A., Leyva, V., Panez, S., Ticona, D., 2007. Frecuencia de *Sarcocystis* sp. en perros pastores de asociaciones alpaqueras de Maragngani, Cusco. Rev Inv. Vet. Peru 18, 84-8.

Dangoudoubiyam, S., Oliveira, J.B., Víquez, C., Gómez-García, A., González, O., Romero, J.J., Kwok, O.C., Dubey, J.P., Howe, D.K., 2011. Detection of antibodies against *Sarcocystis neurona*, *Neospora* spp., and *Toxoplasma gondii* in horses from Costa Rica. J Parasitol. 97, 522-4.

de Costas, S.F., Matas, N.R., Ricoy, G., Sosa, S., Santillan, G., 2014. Diagnosis of cystic echinococcosis in faeces scattered in areas of Puna and Quebrada. Province of Jujuy, Argentina. Rev. Argent. Microbiol. 46, 80-4.

Dittmann, M.T., Runge, U., Lang, R.A., Moser, D., Galeffi, C., Kreuzer, M., Clauss, M., 2014. Methane Emission by Camelids. Plos One 9, e94363.

Dubey, J., Speer, C., Fayer, R., 1989. *Sarcocystis* of animals and man. Boca Raton, CRC Press Inc.

Dufour, E., Goepfert, N., Gutiérrez Léon, B., Chauchat, C., Jordán, R.F., Vásquez Sánchez, S., 2014. Pastoralism in northern Peru during pre-Hispanic times: insights from the Mochica Period (100-800 AD) based on stable isotopic analysis of domestic camelids. PLoSOne 9, e87559.

Echenique, M., Chavez, M., Vittar, M., Longoni, A., 2015. La producción y comercialización de carne de la agricultura familiar en la puna jujeña: análisis de sistemas ganaderos, tramas comerciales y marcos normativos para el diseño de estrategias de desarrollo. INTA Ediciones, Argentina, ISBN 978-987-521-640-2.

FAO, 2005a: Situación Actual de los Camélidos Sudamericanos en Argentina. Available in: Sitio Argentino de Producción Animal, Argentina. http://www.produccion-animal.com.ar/produccion_de_camelidos/camelidos_general/162-situacion.pdf

FAO, 2005b: Situación Actual de los Camélidos Sudamericanos en Perú, 2005. Available in: Universidad Nacional Agraria La Molina, Perú. http://tarwi.lamolina.edu.pe/~emellisho/zootecnia_archivos/situacion%20alpcas%20peru.pdf

Gabor, M., Gabor, L., Srivastava, M., Booth, M., Reece, R., 2010. Chronic myositis in an Australian alpaca (*Lama pacos*) associated with *Sarcocystis* spp. J. Vet. Diagn. Invest. 22, 966-969.

Godoy, R., Vilca, M., Gonzales, A., Leyva, V., Sam, R., 2007. Saneamiento y detoxificacion de carne de llama (*Lama glama*) infectada con *Sarcocystis aucheniae* mediante cocción, horneado, fritura y congelado. Rev. Inv. Vet. Peru 18, 51-56.

Hamidinejat, H., Hekmatimoghaddam, S., Jafari, H., Sazmand, A., Haddad Molayan, P., Derakhshan, L., Mirabdollahi, S., 2013. Prevalence and distribution patterns of *Sarcocystis* in camels (*Camelus dromedarius*) in Yazd province, Iran. J. Parasit. Dis. 37, 163-5.

Howe, D.K., Gaji, R.Y., Mroz-Barrett, M., Gubbels, M.J., Striepen, B., Stamper, S. (2005) *Sarcocystis neurona* merozoites express a family of immunogenic surface antigens that are orthologues of the *Toxoplasma gondii* surface antigens (SAGs) and SAG-related sequences. Infect Immun. 73, 1023-33.

Lamo, D., 2011. Camélidos Sudamericanos: Historia, usos y sanidad animal. Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA). Buenos Aires-Argentina. Cap. 1, 12-22.

La Perle, K.M.D., Silveria, F., Anderson, D.E., Blomme, E.A.G., 1999.Dalmeny disease in an alpaca (*Lama pacos*): sarcocystosis, eosinophilic myositis and abortion. J. Comp. Pathol. 121, 287–293.

Leguía, G., 1991. The epidemiology and economic impact of llama parasites. Parasitol. Today 7, 54-6.

Leguía, G., Casas, E., 1999. Enfermedades parasitarias y atlas parasitológico de camélidos sudamericanos. De Mar E.I.R.L., Lima, Peru, pp. 190.

Leguía, G., Guerrero, C., Sam, R., Chávez, A., 1989. Infección experimental de perros y gatos con micro y macroquistes de *Sarcocystis aucheniae* (*Lama pacos*). Rev. Ciencias Vet. 5, 10–13.

Mamani-Linares, L.W., Gallo, C.B., 2014. Meat quality, proximate composition and muscle fatty acid profile of young llamas (*Lama glama*) supplemented with hay or concentrate during the dry season. MeatScience 96, 394-399.

Marcoppido, G., Vilá, B., 2013. Manejo de Llamas extra-andinas. Observaciones que contribuyen a su bienestar en un contexto no originario. Rev. Arg. .Prod. Anim.. 33, 139-154.

Markemann, A., Valle Zárate, A., 2010.Traditional llama husbandry and breeding management in the Ayopaya region, Bolivia. Trop. Anim. Health Prod. 42, 79-87.

Martin, M., Decker-Franco, C., Romero, S., Carletti, T., Schnittger, L., Florin-Christensen, M., 2016. Molecular detection of *Sarcocystis aucheniae* in the blood of llamas from Argentina. Rev. Argent Microbiol. 48, 200-205.

Medrano, G., Hung, A., Rubio, N., 2006. Detección molecular temprana de *Sarcocystis* en el animal vivo y su estudio filogenético basado en el análisis del gen SSU rRNA en alpacas en Perú. Mosaico Cient. 3, 5-9.

Moré, G., Basso, W., Bacigalupe, D., Venturini, M.C., Venturini, L., 2007. Diagnosis of *Sarcocystis cruzi*, *Neospora caninum*, and *Toxoplasma gondii* infections in cattle.Parasitol. Res. 102, 671–675.

Moré, G., Regensburger, C., Gos, L., Pardini, L., Verma, K., Ctibor, J., Serrano-Martinez, M.E., Dubey, J.P., Venturini, C., 2016.*Sarcocystis masoni*, n. sp. (Apicomplexa: Sarcocystidae), and redescription of *Sarcocystis aucheniae* from llama (*Lama glama*), guanaco (*Lama guanicoe*) and alpaca (*Vicugna pacos*). Parasitology 143, 617-626.

Moré, G., Pardini, L., Basso, W., Marín, R., Bacigalupe, D., Auad, G., Venturini, L., Venturini, M.C., 2008. Seroprevalence of *Neospora caninum*, *Toxoplasma gondii* and *Sarcocystis* sp. in llamas (*Lama glama*) from Jujuy, Argentina. Vet. Parasitol. 155, 158-60.

Regensburger, C., Gos, M.L., Ctibor, J.,Moré, G., 2015.Morphological and molecular characteristics of *Sarcocystis aucheniae* isolated from meat of guanaco (*Lama guanicoe*). J. Food Qual. Haz. Control 2, 118-121.

Rooney, A.L., Limon, G., Vides, H., Cortez, A., Guitian, J., 2014.*Sarcocystis* spp. in llamas (*Lama glama*) in Southern Bolivia: a cross sectional study of the prevalence, risk

factors and loss in income caused by carcass downgrades. Prev. Vet. Med. 116, 296-304.

Saadoun, A., Cabrera, M.C., 2008. A review of the nutritional content and technological parameters of indigenous sources of meat in South America. Meat Sci. 80, 570-81.

StataCorp, 2015. Stata Statistical Software: Release 14. StataCorp LP, CollegeStation, TX.

Wang, L., Copper, R., 2001. Triton X-114 Phase Partitioning for Antigen Characterization. In: Doolan DD (ed) Methods in Molecular Medicine, vol. 72. Human Press, Inc., Totowa NJ, pp. 581-585.

Wheeler J.C., 2012. South American camelids - past, present and future. Journal of Camelid Science 5, 1-24.

Figure legends

Figure 1. Identification and characterization of *Sarcocystis aucheniae* Sa23. Reaction of total protein extracts (a), membrane-bound (b) and soluble proteins (c) of *S. aucheniae* bradyzoites against a *Sarcocystis* spp.-positive llama serum was analyzed by immunoblotting. No bands were observed with *Sarcocysts*-negative serum (not shown). MW protein standards are shown at the left. The black arrow at the right indicates the position of the Sa23 immunoreactive band.

Figure 2. Distribution of seropositivity by Sa23-iELISA in sera of llamas raised under different breeding conditions. Serum samples were collected from llamas bred by itinerant shepherds in the presence of pastoral dogs with no sanitary controls in the Argentine Puna (Group I); in a fenced field, with no pastoral dogs and sanitary controls in the same region (Group II); or in fenced fields, with no pastoral dogs and sanitary controls in the humid Pampas (Group III). The presence of anti-*Sarcocystis* spp. antibodies was tested by the Sa23-iELISA. The graphs show the positivity percentage with respect to a positive reference serum obtained for each individual sample in the three groups. The line shows the position of the cut-off value (31.5% positivity). The number of positive (+) and negative (-) samples that fall above or below the cut-off are shown at the right.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

Highlights

- A soluble ~ 23 kDa antigenic fraction was identified in *Sarcocystis aucheniae* (SA23)
- *Sarcocystis*-seropositivity rates in llamas were determined by SA23-based iELISA
- Seropositivity was significantly higher without than with good management practices
- Geographic location, altitude, climate and pastures did not influence seropositivity
- Gender and age were propensity factors for sarcocystiosis seropositivity of llamas raised under controlled conditions

Figure 1.

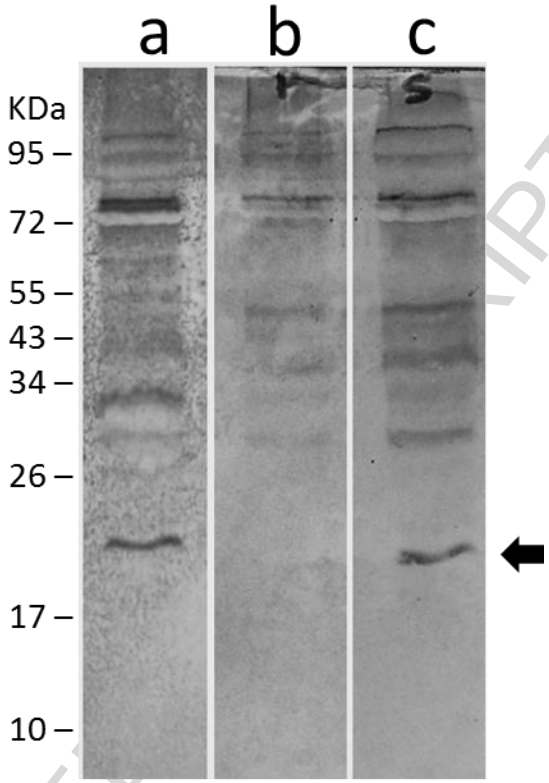
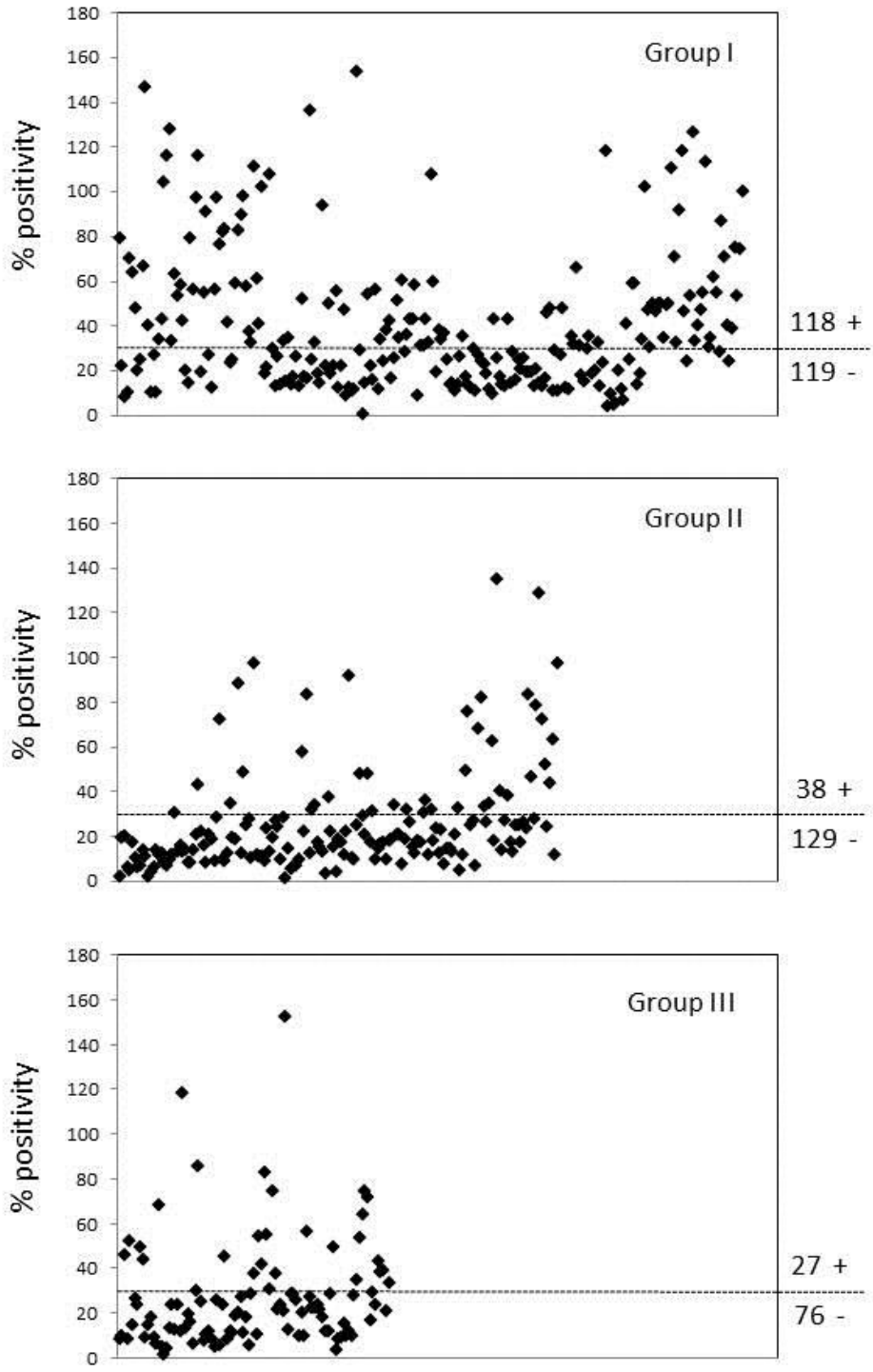


Figure 2.



We declare no conflict of interest regarding the manuscript: ""Seropositivity to *Sarcocystis* infection of llamas correlates with breeding practices".

Monica Florin-Christensen, Ph.D.

Sampling of llamas was carried out following accepted procedures that respect animal welfare.

Monica Florin-Christensen, Ph.D.