Rev. Biol. Trop., 42(3): 623-632, 1994

Seasonal incidence and hemoparasite infection rates of Ixodid ticks (Acari: Ixodidae) detached from cattle in Costa Rica

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(Rec. 27-I-1994. Acep. 12-VI-1994)

Abstract: To determine the tick species hindering the cattle industry in Costa Rica and to assess infection rates of ticks with three important hemoparasite species, cattle were monitored during a period of six months (October 1992 - March 1993). Four farms were located in the dry pacific region of the canton of Tilarán and a fifth farm on the slopes of the Poás volcano in a cool tropical cloud-forest ecosystem. On each farm 3 to 5 animals of 6 to 24 months of age were selected at random. All ticks were removed on a monthly basis from the right half side of each animal, while the site of attachment was recorded. Ticks were counted and differentiated according to species, developmental stage and sex. Moreover, engorged female ticks were assayed for the presence of *Babesia bigemina, Babesia bovis* and *Anaplasma marginale* using the polymerase chain reaction (PCR) multiplex system. Two species of ticks, *Amblyomma cajennense* and *Boophilus microplus,* were encountered on the cattle in the Tilarán region and one species, *B. microplus*, was detected in the Poás region. Two to ten times as many ticks were encountered in the Tilarán region than in the Poás region, which is in accordance with a stable enzootic protozoan disease situation in the former region and an unstable epizootic situation in the latter region. Nymphal and adult stages of both tick species were present in largest numbers on the ventral parts of the animals. PCR analysis of entire ticks indicated very high infection rates with hemoparasites of veterinary importance. This was in accordance with high seroprevalence rates in the hosts.

Key words: Ticks, *Amblyomma, Boophilus,* cattle, hemoparasites, Costa Rica, tick-borne diseases, PCR, ecology.

Ticks and tick-borne diseases cause major losses to the livestock industry in the tropics and subtropics. In Costa Rica, annual losses due to mortality in adult cattle caused by babesiosis and anaplasmosis were calculated to be $64 000 (McCauley and Pérez 1980). Recent investigations measuring the seroprevalence of anaplasmosis and babesiosis indicated that both diseases are ubiquitous in Costa Rica (Pérez *et al.* 1994a). Moreover, seroprevalence varied according to season, ecological life zone and farm management (Pérez *et al.* 1994 a and b).

A country-wide tick survey undertaken by the Ministry of Agriculture and the Food and Agriculture Organization demonstrated that *Boophilus microplus* (Canestrini 1887) was the most important tick species parasitizing cattle and was encountered in nearly the entire territory (Leroy 1980). Furthermore, the report mentioned *Amblyomma cajennense* (Fabricius 1787) to be of importance in cattle in the northern and western parts of the country (Leroy 1980). Little is known, however, about the ecology of these two tick species in Costa Rica nor about the interaction of tick ecology and the epidemiology of tick-borne diseases under local farm management conditions. Recent advances in molecular biology have made it possible using DNA amplification and nucleic acid hybridization methods to determine in a relatively fast and highly sensitive manner the presence of hemoparasites in the tick vectors and ruminant hosts (Fahrimal *et al.* 1992; Figueroa *et al.* 1992). These methods could contribute to a better understanding of the epidemiology of tick-borne diseases and could ultimately provide essential information to

devise more effective vector control and disease prevention strategies.

The objective of the present study was to determine which tick species are most important as parasites of cattle in two major livestock raising regions of Costa Rica, to assess the number and developmental stages of these species during a six-month observation period and to determine tick infection rates with *Babesia bovis* Babès 1888, *Babesia bigemina* Smith and Kilborne 1893 and *Anaplasma marginale* Theiler 1910.

MATERIAL AND METHODS

Tick infestation: Ticks were collected monthly (October 1992 - March 1993) from cattle on farms which were participating in two pilot projects initiated by the School of Veterinary Medicine of the Universidad Nacional. Four farms were situated near the city of Tilarán in Guanacaste province and were representative of the dual-purpose and cow-calf enterprises in that region. The farms had *Bos indicus* cattle and crossbreds of *Bos taurus* x *Bos indicus*. One farm was situated on the slopes of the Poás volcano in Alajuela province and was representative of the specialized dairy production system practised in the highlands of Costa Rica. The farm had Friesian and Jersey dairy cattle. Each farm was located in a different ecological zone (Table 1) as defined by Holdridge (1967).

Daily measurements of minimum and maximum temperatures and rainfall in the two study sites were obtained from the National Metereological Institute in San José (Fig. 1).

At monthly visits three to five animals between six months and two years of age were selected at random. Animals were not to have been treated with acaricides for at least 21 days previous to the visit. The animals were cast on the ground on their left sides and ticks were detached with forceps from the right half of the body. The ticks were collected in plastic vials with perforated lids and differentiated according to its detachment site into five different groups corresponding to regions on the animal (as modified from Gueye *et al.* 1986). The five regions were: (1) ears, (2) head, neck and dewlap, (3) legs and ventral parts of the body, (4) dorsal part of the body and rump and (5) anus, perineum and tail.

Additional information such as exact age of the animal, was extracted afterwards from the data base of the Veterinary Automated Management and Production Program (VAMPP) used as a herd management tool on the farms participating in the two pilot projects (Dwinger *et al.* 1994).

In the laboratory, species, developmental stage and sex of the ticks were determined using a stereomicroscope. The total number of ticks was counted and the number of female *B. microplus* larger than 4.5 mm (being termed "standard" females) was recorded as an indication of engorged tick production per day per

TABLE 1

Characteristics of selected farms on which monthly tick collections were carried out (each row is one farm)

Region	Purpose	Number of cattle	Ecological zone*	Number of animals sampled
Tilarán	cow-calf	887	10	17
Tilarán	double purpose	243	7	26
Tilarán	cow-calf	249	9	20
Tilarán	cow-calf	71	5	12
Poás	dairy	200	15	24

* Ecological zones (Holdridge 1967):
5: Tropical moist forest, premontane belt transition.
7: Tropical wet forest, premontane belt transition.
9: Premontane moist forest, basal belt transition.
10: Premontane wet forest.
15: Lower montane wet forest.

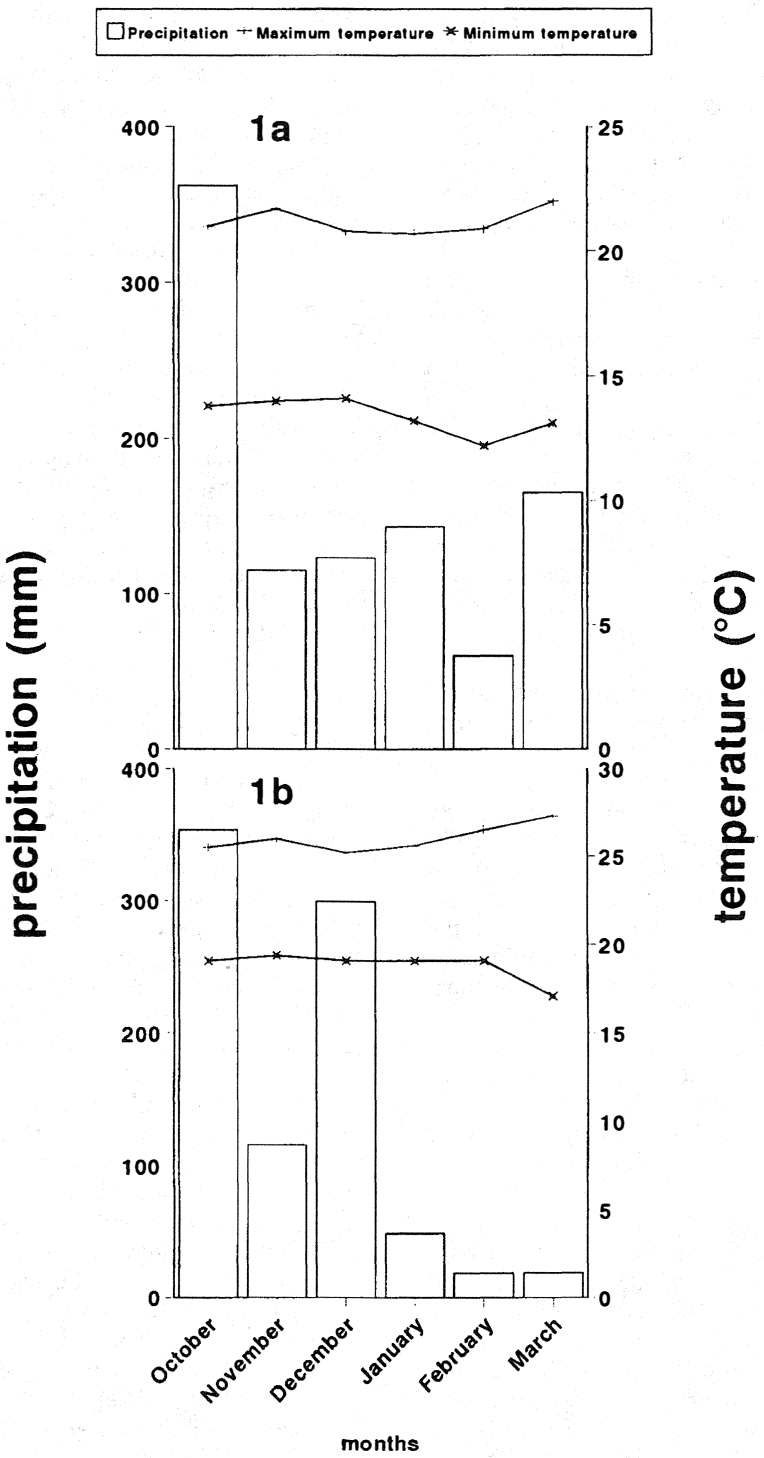

Fig. 1. a) Metereological data from Laguna de Fraijanes on the slopes of the Poás volcano at an altitude of 1850 m (1992-1993). b) Metereological data from San Luis de Tilarán at an altitude of 540 m (1992-1993). Source: Instituto Metereológico Nacional, San José.

animal (Wharton and Utech, 1970). All the female *A. cajennense* were counted independent of size. Numbers were expressed as averages per animal divided by two, since the ticks on half of the animal's body were counted. Following incubation at room temperature and high humidity for at least 24 hours semi-engorged and engorged female ticks were frozen at -20° C.

Host infection rate: From each animal two blood samples were collected from the jugular or coccygeal vein. One blood sample was collected in an empty vacutainer tube in order to separate serum following centrifugation. Sera were stored at -20° C until further analysis. The second blood sample was collected in a vacutainer tube coated with Na-ethylene-diamine-tetra-acetate (EDTA). In addition, a thin blood smear was made by puncturing a capillary ear vein. The smear was fixed in methanol and colored with Giemsa according to standard procedures. Fifty microscopic fields of the slide were inspected for the presence of hemoparasites. The EDTA blood sample was used to determine the packed red cell volume (PCV) using a haematocrit centrifuge.

Serum samples were analyzed with an indirect Enzyme-Linked Immunosorbent Assay (ELISA) for the presence of antibodies against *B. bovis* (FAO/IAEA 1991). In addition a rapid card agglutination test was used to detect antibodies against *A. marginale* according to the instructions provided by the manufacturer (Brewer Diagnostic kit, Wescott and Dunning, Inc.).

Tick infection rate: The presence of three species of hemoparasites was assessed in ticks by using the polymerase chain reaction (PCR) multiplex system as described by Figueroa *et al.* (1993a). To detect *Anaplasma* and *Babesia* parasites in ticks the frozen female *B. microplus* samples were thawed, placed in 1,5 ml microcentrifuge tubes and 490 µl proteinase-K buffer (0,5 M EDTA, pH 8.0; 100 µg/ml proteinase-K; 0.5% sarcosyl) was added. Ticks were allowed to soak in the buffer solution for 30 min and were subsequently disrupted with a clean Pasteur pipet. Next, 20 µl of a 10 µg/µl proteinase-K solution was added. Tick samples were incubated at 65° C for 1 h after which another 20 µl of the proteinase-K solution was added. The tubes were left at 37° C overnight and the next morning mixtures were extracted with a phenol/chloroform/isoamylalcohol mixture (ratio 25:24:1). Nucleic acids were precipitated by adding 200 µl of ammonium acetate (7.5 M) and 1 ml isopropyl alcohol. Following incubation at room temperature for 15 min, samples were centrifuged at maximum speed in an Eppendorf microcentrifuge for 30 min at 4° C. Supernatants were discarded and the pellets were allowed to air-dry.

Crude DNA was purified using the Geneclean system (Geneclean II kit, Bio 101 Inc., La Jolla, CA). DNA pellets were resuspended overnight in 10 µl of dH_2O after which 100 µl saturated NAI and 5 µl silicon particles (glassmilk®) were added. Samples were incubated for 5 min at room temperature and occasionally vortexed. The silicon matrix was spun down in an Eppendorf microcentrifuge at maximum speed for 3 s and the supernatant was discarded. The glassmilk® was washed three times with 200 µl of a cold New Wash® solution (NaCl, Tris, EDTA, ethanol mixture). After the last wash 10 µl dH_2O was added and the sample was eluated at 56° C for 5 min.

Of the eluated sample 2.5 µl was placed in 97.5 µl of PCR reaction mixture buffer containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 mM (each) deoxynucleoside triphosphate (dNTP) (Perkin-Elmer Corp., Norwalk, CT) and 1 mM group I primers for each of the three hemoparasites as described by Figueroa *et al.* (1993b). DNA was denatured by placing the sample at 99.9° C for 10 min. Following the addition of 0.5 µl Taq polymerase (Promega Co., Madison, WI) the tubes were placed in a temperature cycler (Temp.Tronic®, Barnstead/Thermolyne Corporation) going through the following cycles: 2 min of template denaturation at 95° C, 1 min of primer annealing at 60° C and 1.5 min of primer extension at 75° C. The next 35 cycles were identical to the first one except that the template denaturation step lasted only 1 instead of 2 min. The final cycle was a primer extension at 73° C for 13.5 min.

Amplified DNA was hybridized with non-radioactive probes made using group II primers for each of the three hemoparasites as described by Figueroa *et al.* (1993b). The hybridization reactions were detected and visualized by using the dot blot procedure as described by Figueroa *et al.* (1993b). As negative controls 15 non-infected ticks with a aver-

age length of 9.3 mm were assayed in a similar way. Positive controls consisted of serial dilutions of infected erythrocytes for each of the three hemoparasites processed as described by Figueroa *et al.* (1993b).

Statistical analysis: A student t-test was used to test if the numbers of ticks detached from cattle in Tilarán were statistically different from those in Poás.

RESULTS

Tick infestation: Two species of ticks, *B. microplus* and *A. cajennense,* were encountered on cattle in the Tilarán region. *B. microplus* was the only species detected on cattle at the farm in the Poás area.

Tick distribution: Monthly counts of standard female *B. microplus* showed a higher number of ticks to be present on individual animals in Tilarán than in Poás and, thus, total counts to be larger in Tilarán (Fig. 2a). The differences between the two study sites were statistically significant ($P < 0.05$) for the months of December and January. In contrast to the situation in Poás, generally every animal observed in Tilarán was infested with female *B. microplus* (Fig. 2b). The percentage of animals on which adult male and immature stages of *B. microplus* ticks were encountered are not shown, since it can be assumed, *B. microplus* being a one-host tick, that when female ticks were detected the other stages were also present (Wharton and Utech 1970). The monthly distribution of the average number of female *A. cajennense* ticks detached from cattle in Tilarán is shown in Fig. 3a. Fig. 3b shows the percentage of animals infested with the different developmental stages of *A. cajennense.*

Attachment sites: The highest percentage of the different developmental stages of both tick species was encountered on the ventral side of the animals (Fig. 4a and b), except for the larval stage of *A. cajennense* (Fig. 4b). Moreover, larvae of both species were not only detected on the ventral and dorsal side of the body, but also frequently on the ears.

Host infection rate: A total of 86 serum samples were investigated for the presence of antibodies directed against *B. bovis* and *A. marginale,* of which 68 and 18 serum samples came from cattle in Tilarán and Poás, respectively (Table 2). The percentage of cattle with antibodies against *B. bovis* and *A. marginale* ranged from 33% to 100% in the Poás region and from 50% to 100% in the Tilarán region (Table 2). Out of 34 capillary blood smears examined, three were found positive for *A. marginale.*

Tick infection rate: The infection rate of *B. microplus* ticks with *B. bovis, B. bigemina* and *A. marginale* was elevated in both geographical regions (Table 3) and during the months of December and January higher in the Tilarán than in the Poás region (Fig. 5a, 5b and 5c). Non-specific reactions with the *Anaplasma* and the *B. bovis* probe were not detected using the negative tick control samples. However, one non-specific reaction out of the 15 control samples was detected with the *B. bigemina* probe.

DISCUSSION

The average number of ticks collected, as represented by the average number of standard female *B. microplus* ticks, was significantly greater in Tilarán than in Poás during two months of the study period. However, not for the months of November and February due to large standard deviations of the monthly means, which in turn were due to large variations from one farm to another in tick counts of individual animals. Furthermore, during four of the six months studied in Tilarán, all of the sampled animals carried engorged or semi-engorged female ticks and during the other two months 90% or more were infested. In the Poás region, on the other hand, 60% or less of the sampled animals carried engorged or semi-engorged female ticks during four out of the six-month-observation period. *A. cajennense* was not encountered on the cattle in the Poás farm, although Leroy (1980) did report this tick species to be present in the area during a country wide survey. The monthly distribution of the adult forms and instars of *A. cajennense* in Tilarán suggests that not all stages were present throughout the year and that this tick, in contrast to *B. microplus*, has a seasonally defined life cycle in Costa Rica as has been detected previously in Argentina (Guglielmone *et al.* 1990). During the country-wide survey Leroy (1980) encountered sporadically other tick species on cattle in Guanacaste province, such

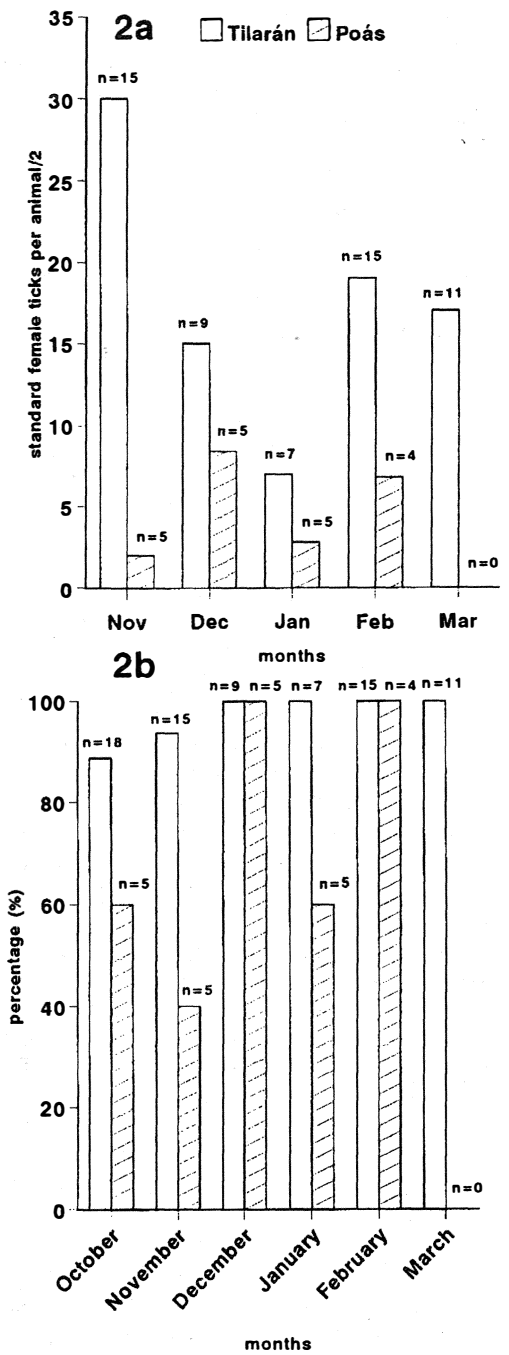

Fig. 2. a) Monthly distribution of the average number of female *Boophilus microplus* ticks (>4.5 mm) per animal (divided by two since ticks were detached from half of the animal) in Tilarán and Poás. b) Monthly percentage of animals infested with female *Boophilus microplus* ticks. n = number of animals monitored.

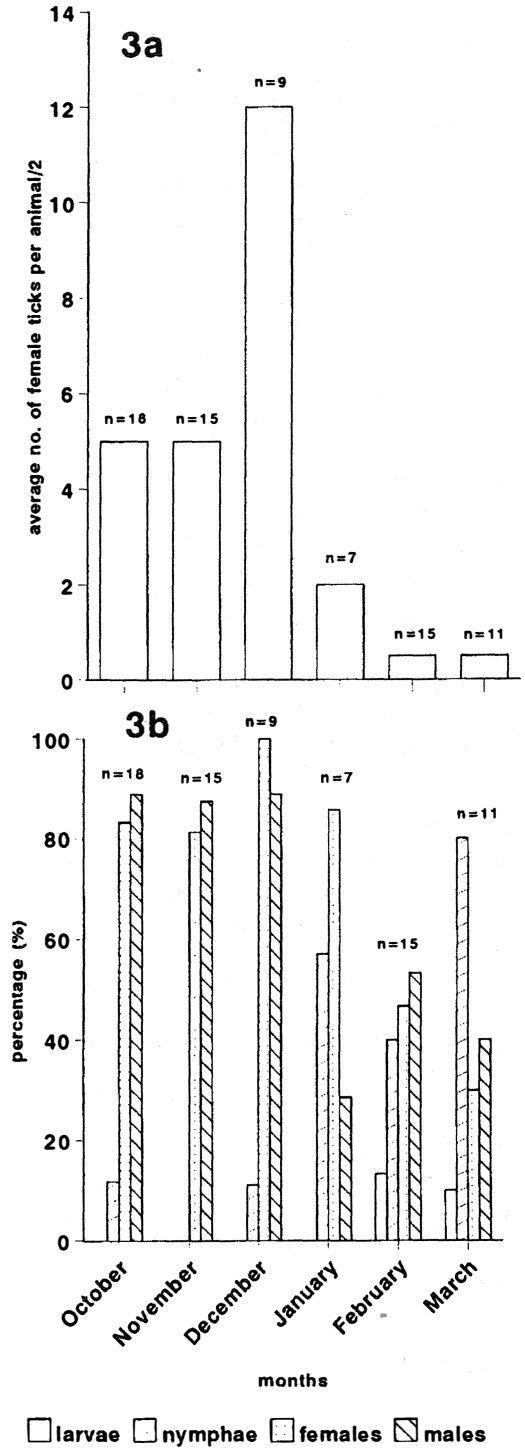

Fig. 3. a) Monthly distribution of the average number of female *Amblyomma cajennense* ticks per animal (divided by two) in Tilarán. b) Monthly percentage of cattle infested with the different developmental stages of *Amblyomma cajennense* in Tilarán. n = number of animals monitored.

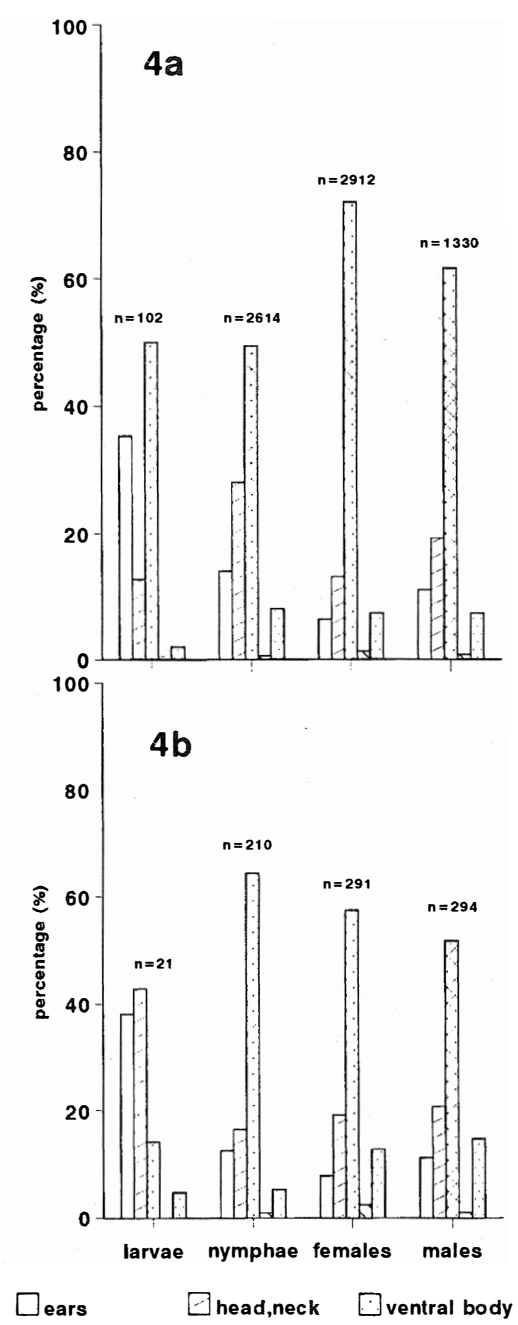

Fig. 4. a) Attachment sites of *Boophilus microplus* ticks collected in Tilarán and Poás and pooled for counting. n = number of ticks counted. Total number of animals monitored: 99. b) Attachment sites of *Amblyomma cajennense* ticks collected in Tilarán. n = number of ticks counted. Total number of animals monitored: 75.

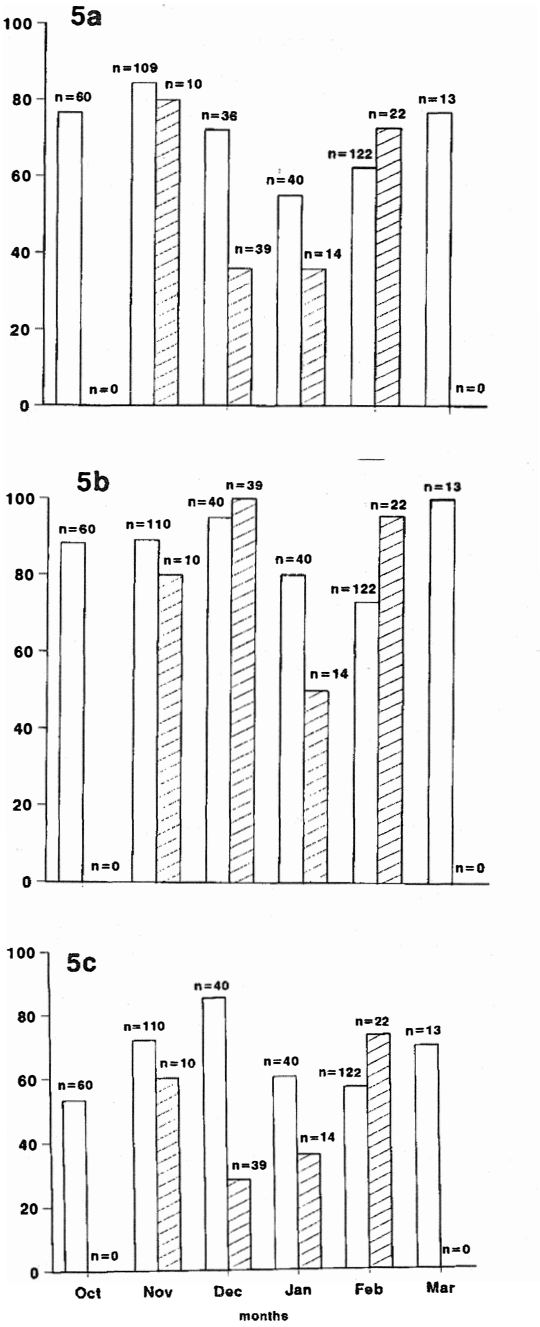

Fig. 5. Infection rates of *Boophilus microplus* with hemoparasites using a polymerase chain reaction multiplex system. Shown are the percentage of ticks infected with a) *Babesia bovis*, b) *Babesia bigemina* and c) *Anaplasma marginale* once detached from cattle in Poás (///) and Tilarán ( ). n = number of ticks investigated.

TABLE 2

Monthly seroprevalence (%) of Babesia bovis and Anaplasma marginale in Tilarán and Poás

Month	Region	*Babesia bovis*	*Anaplasma marginale*
October	Tilarán	9/16* (56.3%)	15/18 (83.3%)
	Poás	5/5 (100%))	3/5 (60%)
November	Tilarán	7/14 (50%)	10/14 (71.4%)
	Poás	-	-
December	Tilarán	8/8 (100%)	8/8 (100%)
	Poás	2/5 (40%)	3/5 (60%)
January	Tilarán	7/7 (100%)	6/7 (85.7%)
	Poás	4/4 (100%)	2/4 (50%)
February	Tilarán	6/12 (50%)	9/11 (81.8%)
	Poás	4/4 (100%)	1/3 (33.3%)
March	Tilarán	10/11 (90.9%)	9/10 (90%)
	Poás	-	-

Overall seroprevalence (%) values of *Babesia bovis* and *Anaplasma marginale* in Tilarán and Poás as compared to the literature.

Region	*Babesia bovis*	Perez *et. al.* (1994b)	*Anaplasma marginale*	Perez *et. al.* (1994b)
Tilarán	47/68 (69%)	59%	57/68 (84%)	57%
Poás	15/18 (83%)	50%	9/17 (53%)	83%

* Example: 9/16 indicates that 9 sera were positive for *Babesia bovis* of the 16 samples investigated using an indirect ELISAtest.

TABLE 3

Infection rates of Boophilus microplus ticks detached from cattle on farms in Tilarán (farm numbers 1,2,3 and 4) and Poás

Month	Farm	*B. bovis*	*B. bigemina*	*A. marginale*
October				
	4	46/60[1] (76.7%)	53/60 (88.3%)	32/60 (53.3%)
November				
	1	41/50 (82.0%)	43/51 (84.3%)	36/51 (70.6%)
	2	31/35 (88.6%)	32/35 (91.4%)	26/35 (74.3%)
	3	11/15 (73.3%)	14/15 (93.3%)	8/15 (53.3%)
	4	9/9 (100%)	9/9 (100%)	9/9 100%)
	Poás	8/10 (80.0%)	8/10 (80.0%)	6/10 (60.0%)
December				
	1	17/24 (70.8%)	27/28 (96.4%)	24/28 (85.7%)
	2	9/12 (75.0%)	11/12 (91.7%)	10/12 (83.3%)
	Poás	14/39 (35.9%)	39/39 (100%)	11/39 (28.2%)
January				
	2	14/29 (48.3%)	22/29 (75.9%)	17/29 (58.6%)
	3	8/11 (72.7%)	10/11 (90.9%)	7/11 (63.6%)
	Poás	5/14 (35.7%)	7/14 (50.0%)	5/14 (35.7%)
February				
	1	8/28 (28.6%)	16/28 (57.1%)	11/28 (39.3%)
	2	30/46 (65.2%)	41/46 (89.1%)	28/46 (60.9%)
	4	30/48 (62.5%)	32/48 (66.7%)	30/48 (62.5%)
	Poás	16/22 (72.7%)	21/22 (95.5%)	16/22 (72.7%)
March				
	3	10/13 (76.9%)	13/13 (100%)	9/13 (69.2%)

1 Example: 46/60 indicates that 46 ticks were positive for *Babesia bovis* of the 60 ticks investigated using the polymerase chain reaction multiplex system. The ticks originated from various bovine hosts.

as *Amblyomma auricularium* (Conil 1878), which parasitizes mainly the armadillo, *Anocentor nitens* (Neumann 1897), primarily a parasite of horses, and *Amblyomma inornatum* (Banks 1909). In another region of Costa Rica, on the slopes of Irazú volcano at an altitude below 2000 m.a.s.l., 99% of the cattle were infested with *B. microplus* and on 1% of the animals *Ixodes boliviensis* Neumann 1904 was detected (Lopez 1976).

In Panama, in addition to the two species mentioned in our report, *Amblyomma oblongoguttatum* Koch 1844, *Amblyomma parvum* Aragâo 1908, *A. nitens* and *I. boliviensis* were encountered on cattle (Fairchild *et al.* 1966).

The majority of the developmental stages of *B. microplus* were detected on the ventral parts of the cattle, although a relatively high percentage of larvae was found on the ears. Nymphs and adults of *A. cajennense* were also primarily found on the ventral parts of the body. As an explanation one might consider that the ventral parts of the body are difficult to groom and form a large exposed area especially when cattle are lying down to ruminate. The larvae of *A. cajennense* seemed to prefer the head, neck, dewlap and the ears of their hosts. This finding agrees with previous reports on *Amblyomma americanum* (Linnaeus 1758) (Barnard *et al.* 1989).

A previous report on the attachment sites of *B. microplus* on cattle grazing the slopes of the Irazú volcano, demonstrated 32% of the ticks to be present on the udder, inguinal and perineal area, 28% in the axillar area, 26% on head and neck and 14% on the ears (Lopez 1976).

Our serological investigations were to corroborate the tick infestation and tick infection rates of the individual animals. Seroprevalence values were in concordance with values obtained by Pérez *et al.* (1994 a), but no epidemiological conclusions could be drawn due to the small sample size.

Infection rates of *B. microplus* with hemoparasites were very high as detected by the sensitive PCR multiplex assay. However, we analysed entire ticks for the presence of infection. Consequently, we can not ascertain whether all ticks found positive, would have transmitted the hemoparasites to the mammalian host. Consequently, it would be more informative to dissect the ticks and subject gut tissue, salivary gland tissue and hemolymph separately to PCR analysis. Stich *et al.* (1993) showed that ticks could be screened for the presence of *A. marginale* with the PCR by testing hemolymph collected from severed legs. Furthermore, experiments on tick transmission of *A. marginale* (Stiller *et al.* 1989) suggested that a minimum time of ten days of tick incubation at 26 °C is needed in order to be able to differentiate between transmissible and non-transmissible strains.

ACKNOWLEDGEMENTS

We are grateful for the financial support from the Ministry of International Cooperation of the Netherlands as part of the interuniversity collaboration between the Universidad Nacional and the State University of Utrecht and for the partial support for the laboratory portion of the project from USAID Prime Agreement DNA 4178-A-00-7056-00. We thank K.K. McLaughlin for technical assistance and personnel of the Proyecto Salud de Hato of the Escuela Medicina Veterinaria for logistic and scientific support.

RESUMEN

Con el objeto de comparar el *status* parasitológico de los bovinos en lo que respecta a garrapatas entre dos regiones de Costa Rica: Tilarán y Poás, se llevo a cabo un muestreo por un periodo de seis meses (Octubre 1992 - Marzo 1993). Cuatro fincas fueron seleccionadas en la región del Pacifico Seco en el cantón de Tilarán y una quinta finca en las faldas del volcán Poás en un ecosistema de bosque tropical nuboso. En cada finca se muestrearon al azar de 3 a 5 animales de una edad comprendida entre los 6 y los 24 meses. Todas las garrapatas fueron removidas mensualmente del lado derecho de cada animal, al mismo tiempo se registró su sitio de adhesión al huésped. Se analizó el número, especie, estadio de desarollo, sexo y sitio de adhesión al hospedero. Las hembras adultas fueron ensayadas para la presencia de *Babesia bigemina, B. bovis* y *Anaplasma marginale* usando el sistema Multiplex de la reacción en cadena de la polimerasa (PCR). Dos especies de garrapatas, *Amblyomma cajennense* y *Boophilus microplus,* se encontraron en bovi-

nos en Tilarán y una especie, *B. microplus,* fue detectada en la región de Poás lo cual sugiere una situación enzoótica estable en la primera región y una situación epizoótica inestable en la última región. Estadios ninfales y adultos de ambas especies de garrapatas estuvieron presentes y en números mayores en las partes ventrales de los animales. Análisis de PCR de garrapatas enteras indicaron altas tasas de infección por hemoparásitos de importancia veterinaria.

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