



Original article

Molecular screening of cattle ticks, tick-borne pathogens and amitraz resistance in ticks of Santo Domingo de los Tsáchilas province in Ecuador

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ABSTRACT

The province of Santo Domingo de los Tsáchilas in Ecuador is a strategic place for cattle production and trade. The lack of knowledge about tick species, tick control and tick-borne diseases in Ecuador prompted this study with the goal of identifying the cattle-tick species and tick-borne agents present in the area and molecularly determining the potential acaricide resistance to amitraz of the major cattle tick species. Eighty-four cattle farms were visited and in 88 % of them, cattle were infested with ticks. Additionally, 24 historical samples from other surrounding Ecuadorian provinces, were screened as well. Besides morphological keys, PCR-RFLP MspI was used to confirm the presence of the *Rhipicephalus* ticks. The tick samples were also screened for tick-borne agents using PCR-RFLP BseDI and HhaI tests to identify circulating *Babesia* sp. and *Anaplasma* spp. Furthermore, the PCR-RFLP EciI technique was used to identify the amitraz resistance gene in populations of *Rhipicephalus microplus* in the province. Pooled testing was used to determine prevalence at individual-tick level. The presence of *R. microplus* and *Amblyomma cajennense* sensu lato (s.l.) ticks was found in 83 % and 21 % of the cattle farms respectively, showing *R. microplus* is widespread in the province of Santo Domingo de los Tsáchilas. Regarding tick-borne agents, only *Anaplasma marginale* was observed in 50 % of the visited farms of the province, while about 27 % of the ticks tested positive according to estimations from the data of the tick pools. The presence of *Babesia bigemina* was only confirmed in samples collected outside the province. The amitraz resistance allele in *R. microplus* was found in 62 % of the farms, but the percentage of farms with cattle ticks completely resistant to this acaricide was low (2%). The findings of this study should prompt cattle producers and animal health authorities to monitor control strategies, which address the management of resistant tick populations and the epidemiologically-unstable areas of tick-borne diseases.

1. Introduction

Ticks are blood-feeding ectoparasites of wild and domestic animals, also occasionally found on people. They occur worldwide and have the capacity of transmitting a variety of pathogens (Guglielmono and Mangold, 2002). The importance of the threat depends on the region, tick species, host populations, the socio-economic situation of livestock producers and the lack of technological progress in the control of these vectors (Solís, 1991). *Rhipicephalus microplus* and *Amblyomma cajennense* sensu lato (s.l.) are the main ectoparasites of cattle present in tropical and subtropical regions of Ecuador. They also represent the major pests of livestock in other South American countries (Nava et al.,

2014).

For cattle, weight loss due to *R. microplus* tick infestation has been estimated at 0.26 kg/tick/year, while for *Amblyomma* spp., 1.09 kg/tick/year (Bayer Healthcare, 2012). *Rhipicephalus microplus* also has a negative impact on milk, meat and hide production (Grisi et al., 2002).

The main tick-borne pathogens transmitted by *R. microplus* are *Anaplasma marginale* (causing anaplasmosis) and *Babesia bovis* and *Babesia bigemina* (causing babesiosis) respectively (Scoles et al., 2011). The epidemiological profile of bovine babesiosis and anaplasmosis depends on factors such as cattle breed and age, climate, stress and pasture management. Three types of areas are recognized in regard to the status of the disease: free, stable and unstable areas. Areas free from

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bovine babesiosis and anaplasmosis are those where conditions are not favorable for development of the vector(s). Stable areas are those where bovine babesiosis and anaplasmosis are present throughout the year, but where animals possess sufficient levels of antibodies to ensure protection. Unstable areas are those with bovine babesiosis and anaplasmosis where the climatic conditions and/or livestock management affect the occurrence of both diseases at certain periods of the year, creating major economic loss due to the lack of immunological protection (Kessler et al., 1983; Barros et al., 2005). Regions where cattle production exists determine the epidemiological risk and the occurrence of outbreaks, as well as the need to implement preventive measures (Amorim et al., 2014).

The use of synthetic acaricides is the primary method of tick control. The presence of ticks resistant to commercial acaricides is a major problem in cattle production in tropical and subtropical areas (Guerrero et al., 2012). Amitraz is an important and widely-used product for the control of cattle ticks around the world, being an affordable, relatively safe and fast-acting acaricide. It has been available for over forty years. Compared to other commercially-available acaricides (with the exception of pyrethroids), amitraz also provides a rapid knockdown effect (Jonsson et al., 2018). Moreover, it offers minimal toxicity for animals and humans, showing a one-day withholding period in meat, two-day withholding period in dairy and rapid breakdown rate. Amitraz has been widely used against cattle ticks in Brazil, especially as an alternative compound for the control of pyrethroid and organophosphate-resistant populations (Mendes et al., 2013). The resistance to amitraz has been well documented in *R. microplus* populations with an increasing trend throughout time (Jonsson et al., 2018). In a study carried out with larval package test (LPT) on twelve Ecuadorian farms, 67 % of the ticks showed phenotypic resistance to amitraz (Rodríguez et al., 2017). Nonetheless, in Ecuador, as in other parts, amitraz continues to be an important product for the control of cattle ticks (Jonsson et al., 2018; Rodríguez et al., 2017).

Santo Domingo de los Tsáchilas has the largest cattle market in the country because of its strategic location. It is here that formal and informal trading of beef, dairy and fattening-calf breeds occurs between the highlands, coastal regions and tropical zones. It has been estimated that about 7500 animals are traded weekly in the formal market. The cattle population in the province alone is estimated at about 200,000 head of cattle (ESPAC, 2017). The major part of the region is tropical, although there are areas in the Andean foothills with a temperate climate. According to the literature, there is a lack of information about the cattle ticks and their related problems in this region, even though there is evidence of acaricide resistance in samples taken on a few farms and where phenotypical tests were used (Rodríguez et al., 2017; Tana-Hernández et al., 2017). The aim of this study is to generate accurate information about the tick species and determine the most important tick-borne diseases that affect cattle in this region, i.e. babesiosis and anaplasmosis, as well as to identify the status of amitraz resistance in the cattle tick *R. microplus* in the herds of this province. The purpose of the study also provides guidance to farmers for better and more effective control strategies.

2. Materials and methods

2.1. Area of study

Tick samples were collected during the rainy season (February – March) in 2012 from 74 out of 84 cattle farms randomly selected from the nine locations that make up Santo Domingo de los Tsáchilas. (Fig. 1). The sampled area was located in the tropical zone of the province at longitudes between 79°25'W and 78°55'W and latitudes between 0°1'N and 0°32'S, at altitudes from 200 to 1500 m above sea level. The tick samples were part of a national survey on brucellosis, tuberculosis and cattle ticks, carried out in mainland Ecuador between 2012 and 2016.

2.2. Tick collection

Ticks were sampled from three parasitized animals chosen from randomly-selected cattle farms. Adults, nymphs, and larvae of *Rhipicephalus* spp. were collected, and for *Amblyomma* spp., only adults were available. In both species, the majority of specimens collected were adults. The number of ticks collected from each animal varied, with at least two specimens taken per animal and placed in 70 % ethanol in a test tube. The tubes were labelled and stored in the laboratory. At sites where ticks were found, sub-samples (pools) of the ticks were obtained for this study. Those sub-samples were divided by species, with 1–10 ticks per test tube, in Santo Domingo de los Tsáchilas (Refer to Supplementary Material No. 1 for detailed information on samples). The remainder were preserved at the Entomology Research Unit of the International Center for Zoonosis (CIZ) of the Central University of Ecuador. Therefore, the average pool size for *Rhipicephalus* spp. was 3.8 ticks, while for *Amblyomma* spp., it was 2.3 ticks. Additionally, another 24 historical samples, containing two to 24 ticks per test tube, from 12 surrounding Ecuadorian provinces from the study area (Carchi, Cotopaxi, Chimborazo, El Oro, Esmeraldas, Guayas, Imbabura, Loja, Los Ríos, Manabí, Santa Elena and Zamora) were also analyzed. These samples correspond to 12 tubes containing *R. microplus*, 9 tubes with *A. cajennense* s.l. and 3 tubes with *Amblyomma maculatum* from farms from the 12 different provinces. These samples were not used for estimating overall prevalence values.

2.3. Morphological identification

A preliminary morphological identification was done and ticks were sorted per species using an Olympus SZ60 Stereomicroscope (zoom range 6.3:1) and dichotomous keys (Jones et al., 1972; Guerrero, 1996; Voltzit, 2007) at the CIZ laboratory. The tick samples were also analyzed at the Institute of Tropical Medicine (ITM), Antwerp in Belgium, and the morphological identification was double-checked using a Discovery V8 Stereomicroscope (Zeiss, Inc.) to identify *Rhipicephalus* and *Amblyomma* at species level, using the morphological keys of Walker et al. (2003) and Nava et al. (2014) as well as the online guide (Madder et al., 2010).

2.4. DNA extraction

A DNeasy® Blood and Tissue Kit (QIAGEN, Hilden, Germany) was used to extract the DNA of the pooled samples. Samples were incubated at 56 °C and 1200 rpm for 2 h for the first trial. Then, they were incubated for 10 min at 56 °C after the first centrifugation. The third incubation lasted 1 min at room temperature (15–25 °C). Finally, the supernatant that contained the DNA was transferred to an Eppendorf tube and was stored at 4.8 °C.

2.5. PCR-RFLP for molecular identification of *R. microplus*

Rhipicephalus microplus molecular identification was performed by PCR, in accordance with protocols of Devos and Geysen (2004) and Lempereur et al. (2010). In cases of positive results, the expected length of the amplified ITS2 gene was 819 bp. RFLP: Positive samples from PCR were selected and submitted to restriction with Msp1 restriction enzyme according to the protocol by Lempereur et al. (2010). The master mix consisted of Ro-Di water, CutSmart® Buffer and Msp1 enzyme. Next, the mixture was vortexed, and 11 µl of this solution were transferred into 1.5-ml tubes with 4 µl of aliquot of the PCR reaction products. The final volume of 15 µl was incubated at 37 °C for 1 h. Five µl of digested solution were mixed with a drop of loading buffer and were transferred into the wells of a 2% high-resolution agarose gel. In the last well of the gel, 2.5 µl of GeneRuler 100 bp DNA ladder were added for fragment-size determination. DNA fragments were spaced through vertical electrophoresis in TBE buffer for 2.5 h at 100 V. Four

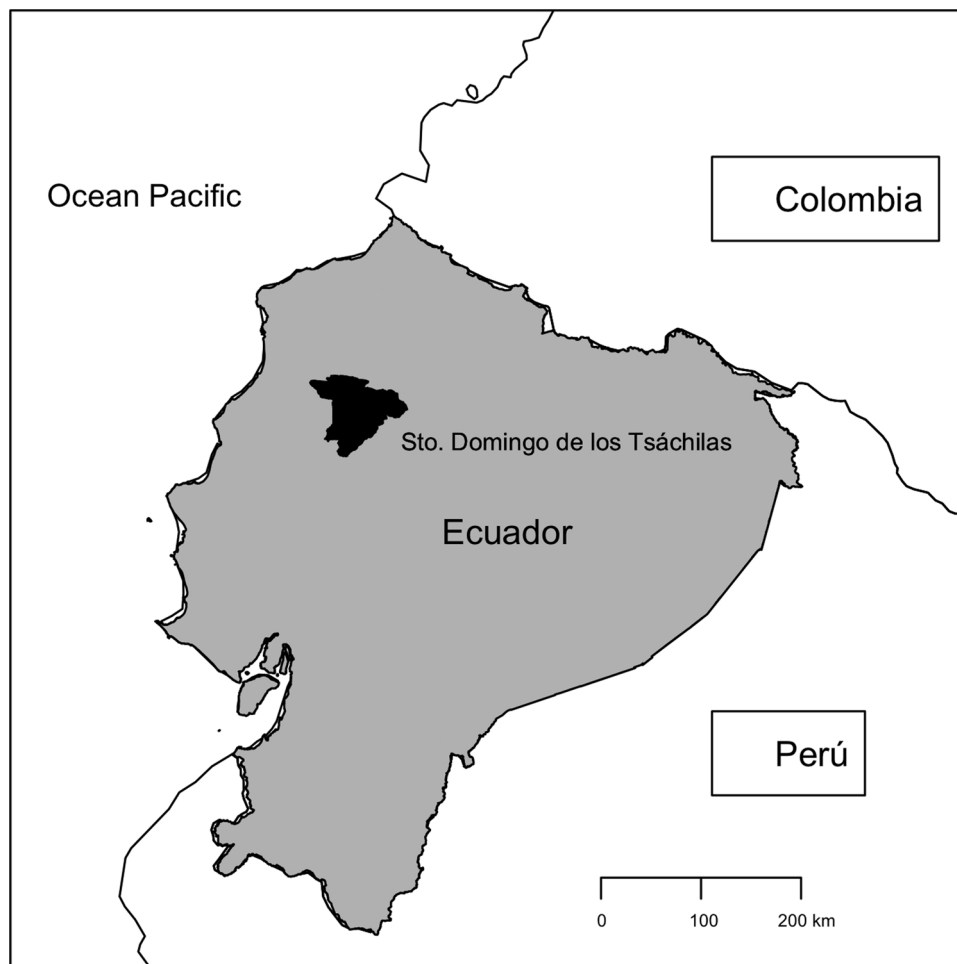


Fig. 1. Santo Domingo de los Tsáchilas (area of study in black).

bands were expected: 421 bp, 241 bp, 97 bp and 70 bp (Devos and Geysen, 2004; Lempereur et al., 2010).

2.6. PCR-RFLP for screening of *B. bigemina*

A nested PCR was run using primer pairs BabF3/R2 (BabF3 5'-ATG TCT AAG TAC AAG CTT TTT ACG GT-3'/ BabR2 5'- TTG ATT TCT CTC AAG GTG CTG AAG GAG TCG-3') for the first round and BabF3/R3 (BabF3 5'-ATG TCT AAG TAC AAG CTT TTT ACG GT -3' / R3 5'-AAA GGC GAC GAC CTC CAA TCC CTA GT -3') for the second round. The expected length was 907 bp (Devos and Geysen, 2004).

Five μ l of extracted DNA were amplified in a 20 μ l of master mix containing Taq Polymerase enzyme (Promega®), the primers (BabF3/R2) and PCR water, resulting in a final volume of 25 μ l of solution. A positive control was used from an *in vitro* culture of *B. bigemina* at ITM as well as a negative control (PCR water). Samples were transferred to PCR Thermocycler T3000. The PCR conditions were 40 cycles of denaturation 30 s at 92 °C, primer annealing 45 s at 62 °C and extension 60 s at 72 °C. For the second round, the master mix contained Taq Polymerase enzyme (Promega®), the primers (BabF3/R3) and PCR water in a total solution of 24.5 μ l per tube. Then, 0.5 μ l of the mixture from the first round were used as template for the second round, resulting in a final volume of 25 μ l. The PCR conditions for the second round were 25 cycles of denaturation 30 s at 92 °C, primer annealing 45 s at 62 °C and extension 60 s at 72 °C. Visualization of PCR products was performed as described in the PCR for molecular identification. Positive samples from Nested PCR were selected and submitted to restriction with BseDI enzyme (Thermo Scientific™) according to the

manufacturer's specifications. The compatible buffer was 10 X buffer TANGO (Thermo Scientific™). The optimal temperature of incubation was 37 °C. In cases of *B. bigemina* detection, the expected bands were 70 bp, 110 bp, 180 bp, 210 bp and 240 bp.

2.7. PCR - RFLP for screening of *Anaplasma marginale*

The laboratory procedures of the PCR protocol for Babesia were also followed for Anaplasma except for the fact that other primers were used. The primers used were EHR 16SD (5'-GGTACCYACAGAAGAAG-TCC-3'), EBR2 (5'-TGCTGACTTGACATCATCCC-3') and EBR3 (5'-TTG TAGTCGCCATTGTAGCAC-3'). For the first round, EHR 16SD/EBR3 was used, while for the second round, EHR 16SD/EBR2 was used (Teshale et al., 2015). The positive control was obtained from an *in vitro* culture of *Ehrlichia ruminantium* and *Anaplasma* spp. at ITM. The negative control used was PCR water.

Positive samples from nested PCR were selected and submitted to restriction with HhaI enzyme (Thermo Scientific™) based on the manufacturer's protocol. The buffer used was 10X Buffer CutSMART (Thermo Scientific™). The temperature of incubation was 37 °C. High resolution agarose gel was used to visualize the digestion. The bands expected were: 538 bp, 99 bp and 84 bp (Teshale et al., 2015).

2.8. PCR-RFLP for screening of amitraz resistance in *R. microplus*

2.8.1. PCR

A single PCR amplification was run using the following primers: OAR-F172 (5'-AGC ATT CTG CGG TTT TCT AC-3) and OAR-R587 (5'-

GCA GAT GAC CAG CAC GTT ACC G-3'). The expected band size of DNA containing the mutations was 417 bp (Baron et al., 2015; Chen et al., 2007). Five µl of extracted DNA were mixed in a 20 µl master mix reaction. The master mix contained Taq Polymerase enzyme (Promega®), 0.2 µM of each primer (OAR-F172/OAR-R587) and PCR water. The samples were transferred to the PCR Thermocycler T3000. The PCR conditions were 40 cycles of denaturation 30 s at 92 °C, primer annealing 45 s at 55 °C and extension 60 s at 72 °C (Baron et al., 2015). The result of the amplification was analyzed on 2% agarose gels with 1% ethidium bromide and observed under UV light TAE buffer. The gel was placed in the electrophoresis machine for 20 min at 100 V.

2.8.2. RFLP

Positive samples from PCR were selected and submitted to restriction with EciI restriction enzyme. The master mix consisted of Ro-Di water, CutSmart® Buffer and EciI enzyme. Subsequently, the mixture was vortexed and 11 µl of this solution were transferred into 1.5-ml tubes with 4 µl of aliquot of the PCR reaction products. The final volume of 15 µl was incubated at 37 °C for 1 h. Five µl of digested solution were mixed with a drop of loading buffer and were transferred onto a 2% high-resolution agarose gel. On the last well of the gel, 2.5 µl GeneRuler 100 bp DNA ladder was added for fragment size determination. The gels were placed in the electrophoresis machine for 40 min at 100 V. Three bands were expected in cases of the mutation leading to amitraz resistance according to Baron et al. (2015): 400 bp, 223 bp and 186 bp.

2.9. Statistical analysis

Our goal was to describe the proportion of infested farms (P), the pathogen prevalence in ticks (π) and the potential percentage of amitraz-resistant gene in ticks (i.e. the tick prevalence estimated from pooled samples). For this purpose, we used a pooled-sample test to generate estimates and credibility intervals (CrI) under Bayesian estimation (Speybroeck et al., 2012). Herds were considered infested when at least either one animal (for tick-infestation) or one tick (for *Anaplasma* spp., *Babesia* sp., and/or amitraz gene presence) was positive. The pathogen prevalence in ticks was calculated by using a Bayesian framework for $\pi = 1 - (1 - P)^{1/kj}$ (Hauck, 1991; Cowling et al., 1999), where kj is the variable pool size per farm (averages: 4 ticks per pool for *R. microplus* and 2 ticks per pool for *A. cajennense* s.l.). We estimated 95 % credibility intervals based on the morphological and molecular results and the posterior distribution of the pathogens prevalence in ticks by using *prevalence* package under R environment (Devleeschauwer et al., 2015). For this proposal, three Monte Carlo chains were generated, and Brooks-Gelman-Rubin (BGR) model statistics was evaluated to be quite near to one to indicate convergence in the estimation.

3. Results

Out of 84 cattle farms, 10 farms did not present cattle ticks. The remaining 74 farms presenting tick specimens were subdivided by species on each farm, giving a total of 69 pools of *R. microplus* and 18 pools of *A. cajennense* s.l. Of those farms, 52 farms had only *R. microplus* and four farms had *A. cajennense* s.l., exclusively. Fourteen farms had both species of ticks. Table 1 and Supplementary Material No.1 describe the obtained results.

3.1. Morphological identification of ticks

Two tick genera and two species were identified morphologically (based on 281 adult ticks, 21 nymphs and 3 larvae): *Rhipicephalus* and *Amblyomma* respectively by genera, and *R. microplus* and *A. cajennense* s.l. by species (Table 1 and Supplementary Material No.1). In Santo Domingo de los Tsáchilas, *R. microplus* was the predominant specimen representing 87 % (95 %CI: 83–91 %) (266/305) of the total ticks

Table 1 Characteristics of ticks collected during the study and comparison of data between Santo Domingo de los Tsáchilas and other Ecuadorian provinces.

Province	Tick prevalence in farms tested ⁻ (%)	Morphological identification of the collected ticks	Number of collected tick stages		Adult male	Nymph	Larva	Average of tick per pool	Percentage of pools tested per tick species ⁺	Tick identification confirmed by molecular tools [†] (%)	Molecular identification of <i>Anaplasma marginale</i> in pools (%)	Presence of the amitraz-resistance allele in pools (%)
			Adult female	Adult male								
Sto. Domingo de los Tsáchilas	88 % (74/84)	<i>Rhipicephalus microplus</i>	164	16	77	21	3	4	82 % (69/84)	99 % (68/69)	60 % (41/69)*	62 % (43/69)
		<i>Amblyomma cajennense</i>	21	21	19	0	0	2	21 % (18/84)	-	6 % (1/18)	-
Other provinces (historical samples)	100 % (24/24)	s.l.	59	59	29	21	2	9	50 % (12/24)	100 % (12/12)	17 % (2/12)	83 % (10/12)
		<i>Rhipicephalus microplus</i>	19	19	18	0	0	4	38 % (9/24)	-	11 % (1/9)	-
		s.l.	3	3	5	0	0	3	13 % (3/24)	-	0	-

⁻ Total number of farms tested in Santo Domingo de los Tsáchilas (n = 84). Total number of farms tested in other provinces (n = 24).
⁺ Total number of pools tested of *R. microplus* (n = 69) and, total number of pools of *A. cajennense* (n = 18) from Santo Domingo de los Tsáchilas province. Total number of pools tested of *R. microplus* (n = 12), total number of pools of *A. cajennense* (n = 9) and, total number of pools of *A. maculatum* (n = 3) from other provinces.
^{*} Three out of forty-one pools showed amplification of both *Anaplasma marginale* and *Anaplasma ovis*.
[†] Tick identification by molecular tools PCR-RFLP (Msp1) was performed afterward morphological identification in pools of *R. microplus*.
[‡] Identification of amitraz resistance genotype in pools was focused in *R. microplus* ticks.
⁻ No testing.

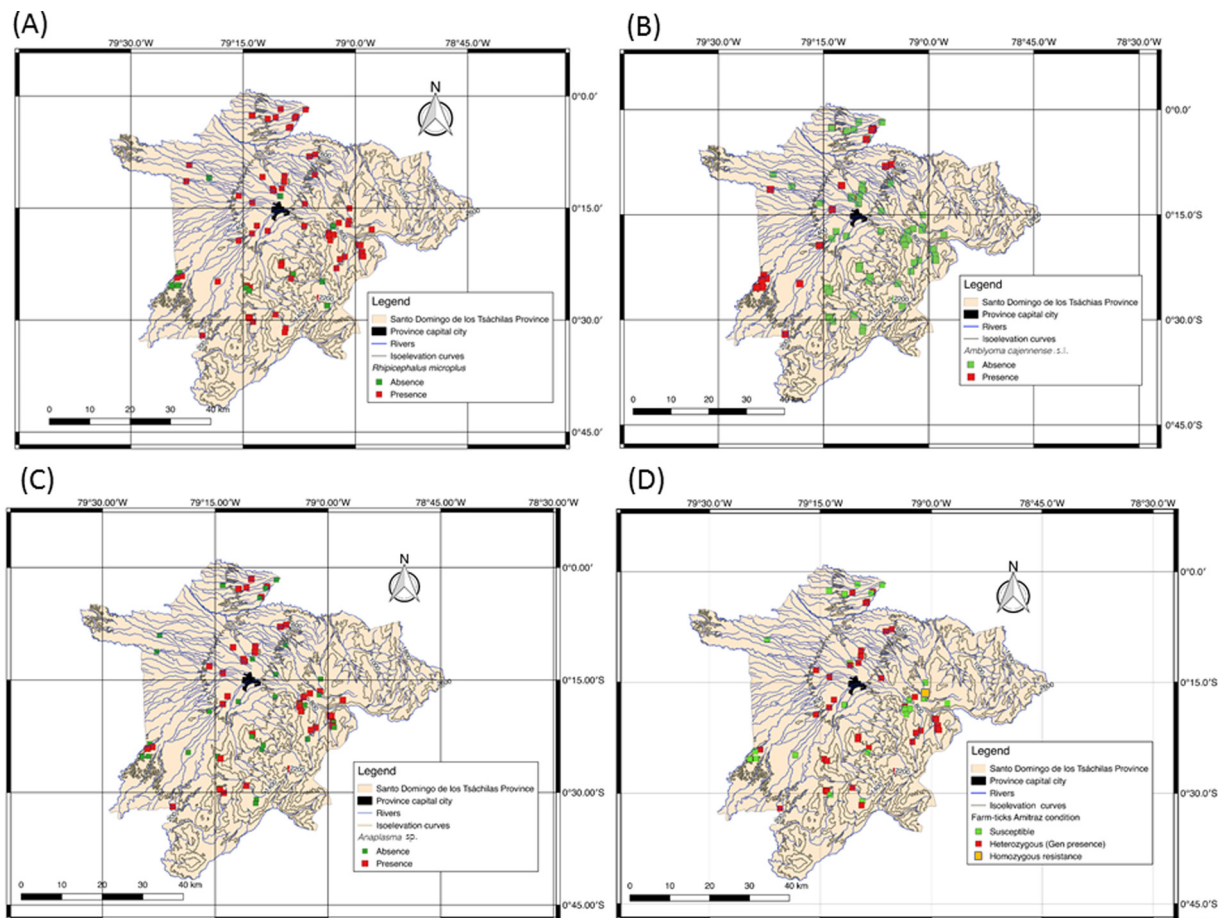


Fig. 2. Spatial distribution of the cattle ticks, tick-borne pathogens and amitraz resistance in the province of Santo Domingo de los Tsáchilas. (A) *Rhipicephalus microplus*, (B) *Amblyomma cajennense* s.l., (C) *Anaplasma* spp. and (D) amitraz resistance.

(Fig. 2A and B), with *A. cajennense* s.l. representing 13 % (95 %CI: 10–17.5 %) (40/305). Four specimens of *Rhipicephalus* sp., one female and three males, were separated from the pools and placed into individual test tubes in order to confirm the species by molecular diagnosis. This was carried out due to the fact that some characteristics did not fit taxonomic patterns (including the presence of the external spurs on the second coxa in females, and the presence of a caudal process in males, which can be similar to the structures of *Rhipicephalus annulatus*) or because the specimen was too small to allow confident identification. For the rest of the country at tick level, using historical samples from the surrounding provinces, *Rhipicephalus (Boophilus)* ticks in the other provinces represented 71 % (111), *A. cajennense* s.l. 24 % (37) and *A. maculatum* 5% (8). The morphological identification of nymphs and larvae was done only at genus level.

At farm level, *A. cajennense* s.l. was found in 21 % of the farms (18/84) (Fig. 2B and Supplementary Material No. 1). It can be seen that *A. cajennense* s.l. increases in presence in the western part of the province of Santo Domingo de los Tsáchilas where climatological conditions are more changeable. On the other hand, *R. microplus* is found on cattle farms throughout the entire province.

3.2. Molecular identification of ticks

Molecular identification was only made for the individuals of the genus *Rhipicephalus*. A total of 69 pools of Santo Domingo de los Tsáchilas and 12 pools of the other provinces was tested (Table 1 and Supplementary Material No.1). In the PCR amplification, 68 samples of the tested samples of Santo Domingo de los Tsáchilas were classified as *Rhipicephalus* sp., 59 amplified in the first analysis and nine samples

amplified in the second test repetition. Only the samples of farm 25 never amplified, although with the surrounding farms, there was confirmation of *Rhipicephalus* sp. All other tick samples from the other provinces were positive for this genus. The four ticks that could not be identified morphologically in the previous step were recognized as *Rhipicephalus* sp.

The RFLP profile of the ticks that were identified as *Rhipicephalus* sp. in PCR showed the profile of *R. microplus*.

Through the molecular identification, it was confirmed that 81 % (95 % CI: 71 %–88 %) (68/84) of the cattle farms in Santo Domingo de los Tsáchilas have *R. microplus*. Fig. 2A shows the spatial distribution of *R. microplus* throughout the province.

3.3. Screening of ticks for Babesia

All ticks of Santo Domingo de los Tsáchilas were found negative for *Babesia* genus. By contrast, a positive sample (0.9 %) for *Babesia* sp. was identified in a *R. microplus* sample coming from El Carchi Province in the north of the country. PCR positive result was confirmed by RFLP which showed that the detected species was *B. bigemina*.

3.4. Screening of ticks for Anaplasma

Of the 84 samples farms tested, nested PCR found 48 positives cases, meaning that the proportion of infected farms was 57 % (95 %CI: 46–68 %) for *Ehrlichia* and/or *Anaplasma* spp. in Santo Domingo de los Tsáchilas (Table 1 and Supplementary Material No. 1).

For the restriction of the PCR product (RFLP), the HhaI restriction enzyme was used on PCR positive samples. Out of 84 samples, 49 %

(41/84, 95 %CI: 37–60 %) were positive for *A. marginale* in *R. microplus* ticks in Santo Domingo de los Tsáchilas Province. For the remaining seven samples, three pools showed amplification of both *A. marginale* and *A. ovis*; one pool showed amplification just for *A. ovis* (Teshale et al., 2015); the other 3 pools never amplified. The prevalence of *A. marginale* in *R. microplus* pools was 58 %. The prevalence in *R. microplus* for *A. marginale* at tick level was 27 % (95 %CrI: 20–35 %).

The prevalence of *A. marginale* in *A. cajennense* s.l. pools was 6% (1 out of 18) (95 %CI: 0–29 %) at herd level, and at tick level, the positivity represented 5% (95 %CrI: 1–14 %). Fig. 2C shows the spatial distribution of *A. marginale* in Santo Domingo de los Tsáchilas.

For the samples coming from other provinces, 13 % of the pools (3/24) were positive for *A. marginale*; 17 % (2/12) were positive in *R. microplus* pools, and 11 % (1/9) in *A. cajennense* s.l.

3.5. Screening of amitraz resistance in *R. microplus* ticks

In Santo Domingo de los Tsáchilas, 67 samples out of 69 *R. microplus* tested pools by single PCR (97 %) were positive for Octopamine/tyramine receptor gene presence (Table 1 and Supplementary Material No. 1). The amplicon was present at 417 bp, as expected. In the pool samples from the other provinces, 100 % amplified for the Octopamine/tyramine receptor gene.

In the RFLP confirmation, one sample (out of 67 pools) presented a unique band at 400 bp, indicating a homozygous genotype and possibly a resistant amitraz phenotype (Baron et al., 2015). This represented 2% of farms (95 %CI: 0–9 %) with genes for amitraz resistance, and confirmed a homozygous genotype prevalence in ticks of 1% (95 %CrI: 0–2 %). Forty-two pools (63 %; 95 %CI: 50–74 %) presented three bands, namely at 400 bp, 223 bp and 186 bp, as a mixture of homozygous and heterozygous ticks, and illustrating the presence of the gene for amitraz resistance (Baron et al., 2015). At the individual level of ticks, the presence of amitraz resistance gene combining homozygous and heterozygous genotypes is about 26 % (95 %CrI: 19–33 %). The 24 remaining samples showed a susceptible pattern of bands. Fig. 2D shows the spatial distribution of the genotypes for the amitraz resistance gene in the province of Santo Domingo de los Tsáchilas.

4. Discussion

Cattle ticks are an important constraint for cattle production in tropical regions. Despite their known economic importance, information on the ecology of ticks, tick-borne pathogens that they transmit and their resistance towards acaricides is often lacking, inaccurate or inadequate in many countries. With this in mind, we focused our attention on the identification of tick species of cattle, the main tick-borne agents they can potentially transmit (*Babesia* sp. and *Anaplasma* spp.) and the amitraz-resistance status of the most important cattle tick (*R. microplus*), in order to gather baseline data in Santo Domingo de los Tsáchilas and ultimately improve cattle health and production. These problems were estimated at farm and at tick levels because they represent an adequate epidemiological description of ticks and tick-related problems in cattle production.

In Santo Domingo de los Tsáchilas, *R. microplus* was found to be the principal tick infesting cattle, recorded on about 83 % of the farms, whereas *A. cajennense* s.l. was found on about 21 % of cattle farms. In the majority of cases, *A. cajennense* s.l. was recorded on farms that were also infested by *R. microplus*. However, this was more prevalent in the western part of the province, where varied seasonal conditions exist, such as temperature and seasonal precipitation, as can be seen in a brief description of the climatological information of the province in the Supplementary Material No. 2 where WorldClim bioclimatic variables (BIO1, BIO2, BIO3, BIO4, BIO7, BIO12 and BIO15) are shown (www.worldclim.org/bioclim). By comparison, *R. microplus* is a more cosmopolitan species and is found on cattle farms throughout the province. The spatial location of the tick species is important in tick-control

management given the fundamentally different lifecycles of the two species. *Rhipicephalus microplus* is a one-host tick and *A. cajennense* s.l. is a three-host tick. Therefore, the control strategies (i.e. treatment and the frequency of application) should be adapted to species ecology. Identification of *Rhipicephalus* (*Boophilus*) spp. ticks is a complicated task due to the fact that the morphological characteristics are very similar across this genus. The difference between females of *R. microplus* and *R. annulatus* is based on the presence of external spurs on the second and third coxae (Lempereur et al., 2010). In males, the caudal appendage in *R. annulatus* is absent. In this study, the caudal appendage on the three males was not very clear. Therefore, a molecular identification, using ITS2 primers for PCR amplification and MspI restriction enzyme, lets us clarify the unique presence of *R. microplus* in the province.

The screening of *B. bigemina* and *A. marginale* in ticks was carried out to determine the prevalence of these agents. These screenings were accomplished by a PCR RFLP tick with high sensitivity. Similarly, this methodology has been used in ticks and blood samples by other authors (Noaman and Shayan, 2010; Teshale et al., 2015). Likewise, this test properly differentiates *Anaplasma* species with high sensitivity. Along the same lines, qPCR would be a useful diagnostic tool to simultaneously screen samples from multiple blood-borne pathogens and for quantifying the burden of these pathogens in the ticks or in the animals. This last test might help to determine the individual tick prevalence and the burden of the bacteria within the ticks (Courtney et al., 2004). Babesiosis and anaplasmosis were taken into account because they are listed as notifiable diseases, due to their socioeconomic impact according to OIE (2008). Furthermore, due to the altitudinal range registered in this province, especially in the eastern part, the conditions for an unstable zone for the bovine babesiosis and anaplasmosis were present since tick populations usually fluctuate considerably due to drastic environmental changes related to altitude and frequent farmer control. Lastly, at present, farmers excessively control ticks because dairy breeds are present in these zones resulting in potentially severe economic losses. *Babesia bigemina* was not found in the ticks of the cattle herds of Santo Domingo de los Tsáchilas. However, the presence of *B. bigemina* in Ecuador can be confirmed, as a sample from Carchi Province in the northern part of the country was positive. *Babesia* and *Anaplasma* infection in ticks is less common than in blood samples of cattle. This statement has been evaluated in different studies where blood samples of cattle in the tropics were tested (Aguayo, 2018; Escobar et al., 2015; Gioia et al., 2018; Tana-Hernández et al., 2017; Oliveira-Sequeira et al., 2005). This information is of interest for disease management given the frequent mobility of animals in the province. The prevalence of *A. marginale* in *R. microplus* pools was about 58 %, leading to an estimated prevalence of 27 % in ticks. These results highlight the necessity of studying the epidemiology of *A. marginale* and its potential reservoirs. The proportion of infected pools with *A. marginale* in *A. cajennense* s.l. was 6%, leading to an estimated prevalence of 5% in *Amblyomma* ticks. Therefore, the major vector in Santo Domingo de los Tsáchilas appeared to be *R. microplus*.

Evaluation of *A. marginale* prevalence at tick level was possible through pooled-sample composition inference. For instance, through this study, we determined that anaplasmosis in the cattle herds using DNA tick information from Santo Domingo de los Tsáchilas is caused by *Anaplasma* spp., where the proportion of infested farms by *A. marginale* was approximately 48 % (95 %CI: 37–60 %), but *A. ovis* and *Anaplasma* spp. were also found in the province. The general prevalence of *A. marginale* considering both species *R. microplus* and *A. cajennense* is 22 % (95 % CrI: 16 %–28 %), giving additional information on spatial risk variation within the province. Conversely, when blood samples from cattle were used, anaplasmosis presented a prevalence of 86.1 % in animals and on 100 % of the studied farms (Tana-Hernández et al., 2017). Pooled-sample testing, in this way, was a cost-effective alternative to testing individual samples, offering the opportunity to produce base-line information, in turn allowing the prioritization of areas

and tick species to be targeted for control.

The use of synthetic acaricides is the primary method of tick control, and the presence of resistant tick lines to commercial acaricides is a major problem in cattle production. Regular monitoring of resistance against different classes of chemical acaricides has an important role in the management of ticks and tick-borne pathogens. According to Chen et al. (2007) and Baron et al. (2015), PCR-RFLP is a good molecular technique to identify the potential amitraz resistance in *R. microplus* ticks. Nevertheless, in Santo de los Tsáchilas, less than 2% of the farms had ticks genotypically resistant to amitraz action, which is considered to be a low proportion of the farm-tick population (compared to 19.4 % found in Mexico by Rodríguez-Vivas et al. (2006)). The presence of the gene for resistance in our study was present in 63 % of the farm-tick population (95 %CI: 50–74 %). Although there was a mixture of homozygous and heterozygous genotypes in farms, if selection pressure continues, it is feared that a real acaricide resistance problem may develop rapidly. At tick level, the presence of amitraz resistance gene, measured by molecular techniques, was about 26 % (95 %CI: 17–31 %), which suggests that the efficacy of the product is reducing. For instance, in a study carried out in an area close to Santo Domingo de los Tsáchilas, as reported by Rodríguez et al. (2017), phenotypic amitraz resistance was found in *R. microplus* in 67 % of the 12 farms using chemical bioassays according to adult immersion test, larval immersion test and larval package test. For the proportion of the farm-tick population, both studies gave similar results concerning to the amitraz resistance. However, they could be measuring different stages (phenotypically or genotypically) of resistance, and the chemical bioassays were not carried out under field conditions. This observation suggests that we must give importance to this problem in cattle areas. Based on this information, monitoring of resistance levels will permit the updating of data and allow for trend anticipation of this emerging problem.

5. Conclusion

In conclusion, *R. microplus* and *A. cajennense* s.l. are the principal tick species present in the province of Santo Domingo de los Tsáchilas, with *R. microplus* being more widespread. The results of morphological identification were corroborated by molecular techniques for the identification of *R. microplus*. *Anaplasma marginale* is very prevalent on farms in the province. However, due to the frequent mobility of animals in the country, *B. bigemina* (found in a neighboring province) should be subject to surveillance. Finally, this screening has confirmed the relatively high frequency of the mutation for Octopamine/tyramine receptor gene, which confers amitraz resistance in ticks, even though there is still a low frequency of tick populations that are completely resistant. Nonetheless, the continued, indiscriminate and incorrect use of this acaricide might increase the selection pressure for resistant phenotypes. These results are helpful in guiding farmers associations in the region to adopt adequate control programs for ticks and tick-borne related problems.

Ethics committee approval

The project was approved and received national consent by the Ministry of Agriculture and Livestock of Ecuador (MAG).

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Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.tbd.2020.101492>.

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