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Original article

# Epidemiology of *Babesia*, *Anaplasma* and *Trypanosoma* species using a new expanded reverse line blot hybridization assay

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ABSTRACT

Vector-borne hemoparasitic infections are a major problem that affects livestock industries worldwide, particularly in tropical and subtropical regions. In this work, a reverse line blot (RLB) hybridization assay was developed for the simultaneous detection and identification of *Anaplasma*, *Babesia* and bovine trypanosomes, encompassing in this way the most relevant hemoparasites that affect cattle. A total of 186 bovine blood samples collected from two different ecoepidemiological regions of northeast Argentina, with and without tick control, were analyzed with this new RLB. High diversity of parasites, such as *Babesia bovis*, *B. bigemina*, *Anaplasma marginale* and three different *Trypanosoma* species, was found. High rates of coinfections were also detected, and significant differences were observed not only in the prevalence of parasites but also in the level of coinfections between the two analyzed areas. Regarding the *Trypanosoma* genus, we provide molecular evidence of the presence of *T. vivax* and *T. theileri* for the first time in Argentina. Besides, since the RLB is a prospective tool, it allowed the identification of a yet unknown bovine trypanosome which could not be assigned to any of the bovine species known so far. In the present study we provide new insights on the prevalence of several pathogens that directly impact on livestock production in Argentina. The RLB assay developed here allows to identify simultaneously numerous pathogenic species which can also be easily expanded to detect other blood borne pathogens. These characteristics make the RLB hybridization assay an essential tool for epidemiological survey of all vector-borne pathogens.

## 1. Introduction

Vector-borne hemoparasitic infections are a major problem that affects livestock industries worldwide, especially in tropical and subtropical regions. In South America, the most important diseases affecting cattle are babesiosis and anaplasmosis, caused by the protozoan parasites *Babesia bovis* and *B. bigemina*, and the rickettsia *Anaplasma marginale,* respectively. Both diseases share clinical signs which include anemia, weight loss, abortions and death (Aubry and Geale, 2011; Bock et al., 2004). In Argentina, the main vector of these pathogens is the tick *Rhipicephalus microplus*, although *A. marginale* is also mechanically transmitted by blood-contaminated fomites or biting flies (Kocan et al., 2010). These parasites are often found together within a single bovine host forming a complex disease known as cattle tick fever. Important economic losses are not only due to mortality and reduced milk and meat production, but also to costs of prevention and treatment of the infected animals.

The eco-epidemiological conditions that favor the development of ticks are also suitable for the presence of other arthropod vectors like biting flies, the main biological vectors of bovine trypanosomes in the Americas. Trypanosomiasis is gaining more importance in South America since several cases in wild and domestic animals have been reported in the last years (Batista et al., 2007; de Araujo Melo et al., 2011; Eberhardt et al., 2014; Galiza et al., 2011; Monzón et al., 2010; Pimentel et al., 2012) and represent a serious drawback for livestock production (Dávila and Silva, 2000). However, considering that most trypanosomiasis cases described in northern Argentina are only based on blood smear observations, there is an urgent need for molecular

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studies to precisely identify which are the circulating species.

The most relevant trypanosome species that affect cattle in America are *T. vivax* and *T. evansi*. The latter is also an important pathogen of horses and water buffaloes and both species cause fever, anemia, weight loss and high mortality rates (Dávila and Silva, 2000). A third species, *T. theileri,* is not considered pathogenic unless associated to other intercurrent infections or stress conditions (Desquesnes, 2005). In South America, where the tsetse flies are not present, trypanosomes have adapted to mechanical transmission by hematophagous arthropods from the *Tabanus* and *Stomoxys* genera (Leunita Sumba et al., 1998; Osório et al., 2008; Otte and Abuabara, 1991). For *T. theileri*, the possible involvement of tick vectors was suggested (Burgdorfer et al., 1973; Latif et al., 2004; Martins et al., 2008; Morzaria et al., 1986; Shastri and Deshpande, 1981).

Although all the pathogens mentioned are considered important by themselves, in natural infections it is common to find multiple parasites within the same host, which is increased by the transmission of the parasites by the same vectors. In this sense, it is crucial to have a single technique to detect all the vector-borne parasites that are expected to be found in the same host. The reverse line blot (RLB) hybridization assay fulfills this aim and has been developed to detect all tick-borne species infecting cattle (Bekker et al., 2002; Gubbels et al., 1999). However, for trypanosome species, individual PCRs have been developed but there are no methodologies to detect and identify all these flagellates at the same time.

The aim of this study was to develop an optimized RLB hybridization assay that includes *Trypanosoma*, *Babesia* and *Anaplasma* species of veterinary relevance which could also help to gather information on the prevalence of vector-borne pathogens in Argentina. The existence of coinfections and unknown parasitic species in the population studied was also analyzed with this strategy.

## 2. Materials and methods

### 2.1. Blood samples and DNA extraction

A total of 186 bovines were sampled from two areas in northeast Argentina selected because of their epidemiologically contrasting conditions: a “tick-control” zone where animals are treated with amitraz every 21 days and no vaccine is used, and a “tick-infested” zone where the trivalent live vaccine (*B. bovis, B. bigemina* and *A. centrale*) is applied and no acaricide treatments are used. The three farms sampled raise beef cattle and Table 1 summarizes the location of the farms and other epidemiologically relevant information such as cattle breeds. Five ml of blood with citrate were collected from each animal and stored at −20 °C. DNA was extracted from 400 µl of blood using the ADN PuriPrep-S kit (INBIO Highway, Argentina) according to the manufacturer’s instructions and eluted in 70 µl of elution buffer. DNA was quantified using ThermoScientific Nano Drop 1000 (Thermo Fisher Scientific, USA) and stored at −20 °C.

**Table 1**
Location and number of animals in the farms sampled.

| | Farm | Location | Area | Number of samples | Bovine Breeds |
|---|---|---|---|---|---|
| 1 | La Salvación (LS) | Pilcomayo, Formosa 25°27′62.3″S 57°51′17.3″W | Tick-infested | 38 calves | Senepol |
| 2 | Santa María (SM) | Pilcomayo, Formosa 25°24′23.0″S 58°07′02.5″W | Tick-infested | 39 calves, 15 adult cows | Argentine criollo |
| 3 | EEA Mercedes (EEAM) | Mercedes, Corrientes 29°11′46.5″S 58°02′35.4″W | Tick-control | 94 calves | Braford |

### 2.2. Parasite strains used in this study

DNA from different reference sources was used as positive controls. Genomic DNA of *T. vivax* strain “TvMi” was kindly provided by Dr. Fernando Paiva from Universidade Federal do Mato Grosso do Sul, Brazil. DNA from *T. theileri* was obtained from a natural infected bovine from this study. This sample was confirmed as *T. theileri* by sequencing of the V7V8 fragment of the 18S rRNA gene and deposited in GenBank under accession number KY009582. *T. evansi* genomic DNA was from an infected equine that arrived at the Centro de Investigaciones y Transferencia Formosa, Argentina and was provided by Vet. Fernando Dubois. Reference strains of *B. bovis, B. bigemina, A. marginale* and *A. centrale* were previously characterized (Guillemi et al., 2015, 2013).

### 2.3. Primer and probe design

The RLB assays previously developed by Bekker et al. (2002) and Gubbels et al. (1999) based on the 16S and 18S rRNA genes for the identification of *Anaplasma/Ehrlichia* and *Babesia/Theileria* respectively, were used including additional probes previously developed by our group to detect new *B. bigemina* genotypes present in Argentina (Petrigh et al., 2008). This RLB membrane was further expanded to detect relevant bovine trypanosomes such as *T. vivax*, *T. evansi* and *T. theileri*. For probe design, sequences of the 18S rRNA gene from different trypanosome species that were reported to be present in America (Dávila and Silva, 2000; Silva et al., 2002) were retrieved from the GenBank database and aligned using the Clustal Ω algorithm (www.ebi.ac.uk/Tools/msa/clustalo/). PCR primers were manually designed to amplify the V7V8 portion of the gene of all trypanosome species and a genus-specific probe was also designed in a conserved region. Additionally, species-specific probes for *T. evansi*, *T. theileri* and *T. vivax* were selected in a hypervariable region. Sequences of all available strains of these trypanosome species were aligned and oligonucleotides were designed in conserved regions among the different strains. A fourth species-specific probe (*T.* sp. Formosa) was developed after observing that for some samples the genus-specific probe detected a positive signal but the species-specific probes failed to identify these samples. The V7V8 region was amplified by PCR in these samples, sequenced and the new probe was designed based on this information.

All oligonucleotides used in this study are listed in Tables 2 and 3 and were checked for specificity and melting temperatures, together with the potential for self and hetero-dimer formation, using the IDT-DNA Oligoanalyzer tool (https://www.idtdna.com/calc/analyzer). In order to rule out cross-reaction of the probes, an alignment of the portion of the 18S rRNA gene where all the eukaryotic species-specific probes hybridize (V4 region for *Babesia* and V7V8 region for *Trypanosoma*) was performed (see Supplementary Fig. S1).

### 2.4. PCR amplification and sequencing

For the RLB hybridization assay, PCR reactions described by Bekker et al. (2002) for *Anaplasma/Ehrlichia* (AE-PCR) and Gubbels et al. (1999) for *Babesia/Theileria* (BT-PCR) were modified by including a second step of PCR amplification to increase the sensitivity of detection of all species (Schnittger et al., 2004; Tomassone et al., 2008). To amplify the 18S rRNA gene of *Trypanosoma* spp., a set of two external primers and one internal biotin-labeled primer was designed and a hemi-nested PCR was set up (T-PCR). For the first round, the amplification was performed in a 20 µl reaction mixture containing 0.4 µM of each external primer, 0.2 mM of each deoxyribonucleotide triphosphate (INBIO Highway, Argentina), 0.5 U of GoTaq DNA polymerase (Promega, USA), 1X PCR buffer and ~50 ng of genomic DNA. Cycling conditions included an initial 3 min denaturation at 94 °C, followed by 20 cycles, which consisted of denaturation at 94 °C for 30 s, annealing

**Table 2**
Primers for hemi-nested PCR amplification of the rRNA genes.

| Genus | Primer | Sequence (5′ → 3′) | Reference |
|---|---|---|---|
| *Babesia/Theileria* (BT-PCR) | RLB-F | GAGGTAGTGACAAGAAATAACAATA | (Gubbels et al., 1999) |
| | RLB-Fint | GACAAGAAATAACAATACRGGGC | (Schnittger et al., 2004) |
| | RLB-R | /5Biosg/TCTTCGATCCCCTAACTTTC | (Gubbels et al., 1999) |
| *Anaplasma/Ehrlichia* (AE-PCR) | HER-F | AGAGTTGGATCMTGGYTCAG | (Bergmans et al., 1995) |
| | HER-Fint | GGCTCAGAACGAACGCTG | (Tomassone et al., 2008) |
| | HER-R | /5Biosg/CGGGATCCCCAGTTTGCCGGGACTTYTTCT | (Bekker et al., 2002) |
| *Trypanosoma* (T-PCR) | 18STryp-F | GGTAATTCCAGCTCCAAAAGCGT | This study |
| | 18STryp-Rint | /5Biosg/TCCTTGAAGAATGCCTTCGCTGT | This study |
| | 18STryp-R | GCAGTGTGGACTACAATGGTC | This study |

at 62 °C for 45 s and elongation at 72 °C for 1 min, followed by a final extension step of 72 °C for 10 min. The second step was carried out in a 50 µl reaction mixture using as template 3 µl of the product of the first step. Reaction mixture contained 0.4 µM of each internal primer (one of them biotin-labeled), 0.2 mM of each deoxyribonucleotide triphosphate, 1.25 U of GoTaq DNA polymerase and 1X PCR buffer. Reaction conditions comprised an initial denaturation step of 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 65 °C for 45 s and extension (72 °C for 1 min). A final extension at 72 °C for 10 min was performed.

T-PCR amplicons of approximately 550 bp were purified using QIAquick PCR purification kit (Qiagen, Germany). Products were sequenced in both strands using the same PCR primers and the Big Dye Terminator v3.1 kit from Applied Biosystems and analyzed on an ABI 3130XL genetic analyzer from the same supplier. Chromatograms were manually analyzed and did not show double peaks that would indicate mixed infections.

### 2.5. Reverse line blot hybridization assay

For the setup of the *Trypanosome* membrane, two probe concentrations were tested (200 and 100 pmol/lane). Since all showed the same intensity of signal (data not shown), the highest concentration was maintained to warrant high sensitivity of the membrane.

For each sample, three biotin-labeled PCR products were generated, corresponding to the BT-PCR, T-PCR, and AE-PCR and mixed in equal volumes (40 µl of each PCR product were mixed and brought to a final volume of 150 µl with SSPE 0.1% SDS buffer) prior to be applied to the membrane. All subsequent procedures were done as described before by Bekker et al. (2002).

### 2.6. Statistical analysis

Chi-square test calculations were used to statistically evaluate the influence of location on the observed parasite prevalence. $P < 0.05$ was accepted to be statistically significant. A correlation matrix to assess significant associations between species was generated using the Pearson's method. All analyses were conducted using the Infostat software.

### 2.7. Phylogenetic analysis

Alignments of V7V8 region of the *Trypanosome* 18S rRNA were conducted with ClustalW and phylogenetic analyses were performed with the Maximum Likelihood method based on the Kimura 2-parameter model, using *T. cruzi* as outgroup. Only samples that yield sequences of acceptable quality and length were included in the construction of the phylogenetic tree. The topology of the tree obtained with Maximum Likelihood was further compared with the tree obtained with the Neighbor Joining method. In all cases bootstrap values were calculated with 1000 replicates. Analyses were done with MEGA 6.0 software (Tamura et al., 2013).

## 3. Results

### 3.1. RLB development for bovine trypanosomes

Complete sequences of the 18S rRNA gene from different trypanosome species of bovines, equines and humans available in GenBank were aligned and the V7V8 hypervariable region was selected for designing species-specific oligonucleotide probes for *T. evansi*, *T. theileri* and *T. vivax*. Additionally, a conserved region among all the species of the genus was selected for designing a genus-specific probe of 28 bp (Fig. 1).

Specificity of the T-PCR reaction using 18STryp-F and 18STryp-R primers was evaluated with control DNA from *T. theileri* and *T. vivax,* and also with DNA from *B. bovis, B. bigemina* and *A. marginale* which may be found within the same mammalian host. In the cycling

**Table 3**
Genus and species-specific probes used for the RLB hybridization.

| Probes | pmoles/lane | Sequence (5′ → 3′) | Reference |
|---|---|---|---|
| *Babesia/Theileria* genus probe | 100 | CTGTCAGAGGTGAAATTCT | (Gubbels et al., 1999) |
| *B. bovis* | 200 | CAGGTTTCGCCTGTATAATTGAG | (Gubbels et al., 1999) |
| *B. bigemina* | 100 | TCTTTTCGCTGGCTTTTTTTTTA | (Petrigh et al., 2008) |
| *B. bigemina* M | 200 | CGTTTTTTCCCTTTTGTTGG | (Petrigh et al., 2008) |
| *B. bigemina* P | 200 | CGTTTTTTCCCTCTTTTCGG | (Petrigh et al., 2008) |
| *B. bigemina* A | 200 | CGTTTTTTCCCTGGTTTTGG | (Petrigh et al., 2008) |
| *B. bigemina* T | 200 | CGTTTTTTCCCTCGTTTTGG | (Petrigh et al., 2008) |
| *Anaplasma/Ehrlichia* genus probe | 100 | GGGGGAAAGATTTATCGCTA | (Bekker et al., 2002) |
| *A. marginale* | 200 | GACCGTATACGCAGCTTG | (Bekker et al., 2002) |
| *A. centrale* | 200 | TCGAACGGACCATACGC | (Bekker et al., 2002) |
| *Trypanosoma* genus probe | 200 | GCTGTTGCTGTTAAAGGGTTCGTAGTTG | This study |
| *T. evansi* | 200 | ATTCATTGCGACGCGCGGCTTCCA | This study |
| *T. theileri* | 200 | CCTTCTCGTGACTCACGGCATCCA | This study |
| *T. vivax* | 200 | GCATTCCACTCGGAACACGCCGCA | This study |
| *T. sp* (Formosa) | 200 | TGAGCTGTTTGGTATCGGGGTCCA | This study |

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T. evansi        GCGTATATTAATGCTGTTGCTGTA-AAGGGTTCGTAATTGAACTGTGGGCCACGTAGTTTTGTGCCG-TGCCA
T. theileri      GCGTATATTAATGCTGTTGCTGTTAAAGGGTTCGTAGTTGAATTGTGGGCCTTTGAGGCG----CAATGGTTT
T. vivax         GCGTATATTAATGCTGTTGCTGTTAAAGGGTTCGTAGTTGAACTGTGGGCCGCGCTGCCG----CCGCGCTCC
T. sp Formosa    GCGTATATTAACGCTGTTGCTGTTAAAGGGTTCGTAGTTGAATTTTGAGCTGTTTGGTAT----CGGGGTCCA
                 *********** ************ ***************** * ** **      *       *

T. evansi        GTCCCGTCCCCCTCGGACGTGTT--TTTGACCC-ACGCCCTCGTGGCCCGTGAACACACTCAGATACAAAAAA
T. theileri      GTCCCGTCCACCTCGGATTG------GTGACCC-ATGCCCTTGAGGTCCGTGAACAA--TCAGAAACAAAAAA
T. vivax         GCCC------CACCCGCAGAGGGTGCGTGGCACGCGGCGCGCGCTGCCCGCGAACAC--TCAGACACAAGCAG
T. sp Formosa    GTCCACTTCACTACTGAAGTGGG--CTT------CCCCACCTTCAGCTCGAGAACA---TCAGAAACAAATAC
                 * **      *  * *           *         *       *  ** *****   ***** ****  *

T. evansi        -ACCGGAGCGGGTCCTCTCACTTTCACGCATGTCATGCATGCGAGGGGGCGTCCGTGAA-TTTTACTGTGACC
T. theileri      CACGGGAGCGGTTCCTTCCTGATTTTCGCATGTCATGCATGCCAGGGGGCGTCCGTGAT-TTTTACTGTGACT
T. vivax         CGCGGGAACGC---TTCCCCACACCACGCGTGTCATACACGCACGGG-GCGCCCGCGAGTCTTTACTGTGACT
T. sp Formosa    CACGGAATTGTATTTTCCCCCGGATTCGCGGTCAATGCCCGCAGGGGGACGTCCGT-GTTCATTACTGTGACT
                   *** *  *     *  *       ***     ** *  **  ***  ** ***       **********

T. evansi        AAAAAAGTGCGACCAAAGCAGTCTGCCGACTTGAGTTACAAAGCATGGGATAACGAAGCATCAGCCCTGGGGC
T. theileri      AAAAAAGTGTGACCAAAGCAGTCATTCGACTTGAATTAGAAAGCATGGGATAACAAAGGAGCAGCCTATGGGC
T. vivax         AAAAAAGCGCGACCAAAGCAGTCCACCGACCTGAATTGCAAAGCATGGGATAACAAAGCATCAGCCTCCGGGC
T. sp Formosa    AAAAAAGTGCGACCAAAGCAGTCCAACGACTTGAATTAGAAAGCATGGGATAACAAAGGAGCAGCCTACCGGC
                 ******* * *************   **** *** **  *************** *** * *****    ***

T. evansi        CACCGTTTCGGCTTTTGTTGGTTTTAGAAGTCCTTGGGAGATTATGGGGCCGCGTGCCTT-GGGTCGGTGTTT
T. theileri      CACCGTTTCGGCTTTTGTTGGTTTTAAAAGTCCATTGGAGATTATGGCGTCGTGCGACA-AGCGTCTGGGTGT
T. vivax         CACCGATTCGGCTTTTGTTGGTTTTAACAGTCCGTTGGAGATTATGGGCCGGCGCGAGCACGCACCG-CG-GC
T. sp Formosa    TACCGTTTCGGCTTTTGTTGGTTTTAACAGTCTGTGGGAGATTATGGACGGTCAGGACCG-GTGTTG-GG-GG
                  **** ********************  ****  * ***********

T. evansi        CGTGTCTCATTTTTGTGGCGCGCACATTC-----G-GCTCTTCGTGATGTTTTTTT----------ACATTCA
T. theileri      --TTTCCCATCTTTGGGGCACCCGTCGCCTTTGCGAGAAATCCGTGTCGGTGTTGATGAGCTTCGGCCCATCT
T. vivax         --ATTCCACTCGGA-----ACACGCCGCA--------CGCCCCGCGGCG-CC---TCGCGCGTCG--------
T. sp Formosa    --TTTACCCTCTT-------CACTTCT-------------CCTGCGTTGGACTTGTCCAGCGTTA--------
                     *    *           *                   *

T. evansi        -------------TTGCGACGCGCGGC-TTCCAGGAATGAA
T. theileri      CTCCGGCGCCTTCTCGTGACTCACGGC-ATCCAGGAATGAA
T. vivax         --------------C--GG--CTCGCCGGCCCGGGAATGAA
T. sp Formosa    --------------G-GGGCCTGCGCCGTTC-AGGAATGAA
                               *     ** *   *  ********
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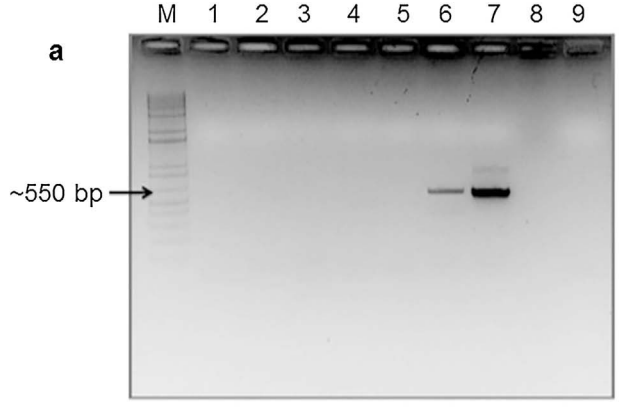
**Fig. 1.** Alignment of the V7V8 hypervariable region of the 18S rRNA gene of different *Trypanosoma* species. Location of genus- (shaded) and species- (underlined) specific probes are shown. Conserved positions among sequences are indicated by asterisks.



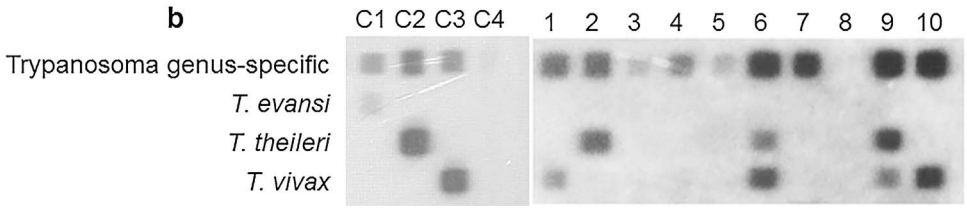




**Fig. 2.** Specificity of the *Trypanosoma* sp. primers and probes. a) Agarose gel electrophoresis of amplified DNA from different *Trypanosoma*, *Babesia* and *Anaplasma* species using the T-PCR. M, molecular size marker (1Kb Plus DNA Ladder); lane 1, uninfected bovine; 2, *B. bovis*; 3, *B. bigemina*; 4, *A. marginale*; 5, *A. centrale*; 6, *T. theileri*; 7, *T. vivax*; 8, empty lane; 9, negative control (water). Arrow indicates the expected amplicon size. b) Reverse line blotting of PCR products obtained with the T-PCR from controls and a selection of 8 field samples to exemplify the diverse results that were obtained. Probes were applied in horizontal lines. C1, *T. evansi* positive control; C2, *T. theileri* positive control; C3, *T. vivax* positive control; C4 negative control. Lane 1, *T. vivax* positive control; 2, *T. theileri* positive control; 3–10, field samples.

conditions set, amplicons of the expected size were only observed for *T. theileri* and *T. vivax* (Fig. 2a). In addition, to evaluate the specificity of the new oligonucleotide probes for trypanosome species, the products generated with the T-PCR were hybridized to the membrane where the probes had been previously fixed. In all the cases a positive signal was only observed with the correct genus- and species-specific probes and there was no unspecific reaction with the heterologous DNA. Reverse line blotting of results obtained with the T-PCR from 2 positive controls of *T. vivax* and *T. theileri* and 8 field samples are illustrated in Fig. 2b. This figure shows a negative sample (lane 8), a sample with only one species (lane 10), two samples with coinfections (lanes 6 and 9) and four samples (lanes 3, 4, 5 and 7) that only hybridized with the *Trypanosoma* genus probe but not with the species specific probes.

With the aim of gaining deeper information from the results obtained with the RLB for trypanosomes, the V7V8 region of all field samples was amplified by PCR with the 18STryp-F and 18STryp-R primers, sequenced and species identity was determined by BLAST search. These results were then compared with the species identification obtained with the RLB. Interestingly, there was a group of samples from the Formosa region that only hybridized with the *Trypanosome* genus-specific probe and failed to hybridize with any of the species-specific probes (Fig. 2b, lanes 3–5 and 7). Therefore, the products of the T-PCR were sequenced and found to be identical to each other but different to *T. evansi*, *T. theileri* and *T. vivax*. Nucleotide BLAST searches of these sequences of 529 bp against GenBank were done and the best match obtained showed a 91% of nucleotide identity (e-value 1e-180) that aligned with 92% of the query sequence of a 18S rRNA sequence of an uncultured trypanosome from an environmental sample (GenBank accession number JN629293). This new sequence was provisionally named "*T.* sp. Formosa" and was added to GenBank under accession number KY009571.

Based on these results, a new oligonucleotide probe specific for *T.* sp. Formosa was designed 5 bp downstream of the genus-specific probe (Fig. 1) and added to the RLB membrane. Afterwards, the specificity of this new probe was tested as above mentioned and, as expected, showed a positive signal only in the samples containing this new variant.

### 3.2. Field samples analyzed with RLB

A total of 186 blood samples collected from two different eco-epidemiological regions (Fig. 3) were analyzed by RLB. Three independent PCR reactions were conducted per sample and the T-PCR, AE-PCR and BT-PCR products were mixed prior to the hybridization on the membrane (Supplementary Fig. S2). With the aim of testing the assaýs reproducibility, the RLB was repeated twice in the same group of 80 samples (43% of the total samples) and both independent assays showed identical results. The overall results showed that 157 (84.4%) samples were infected with at least one hemoparasite. A total of seven different species were found in the study area (Table 4). The most prevalent hemoparasite in La Salvación (LS) was *A. marginale* (71.1%) whereas in Santa María (SM) both *A. marginale* and *T. theileri* (46.3%) were the most prevalent. In EEA Mercedes (EEAM) instead, the species most frequently found was *B. bigemina* (58.5%). *T. evansi* was never detected in any of the farms.

The prevalence for each individual parasite showed significant differences between the two areas analyzed ($p < 0.05$). However, between the two farms of Formosa without tick-control (LS and SM), significant differences appeared only in the prevalence of *A. marginale* ($p = 0.02$), *T.vivax* ($p < 0.005$) and *T.* sp. Formosa ($p < 0.001$) (Table 4 and Supplementary Table S1). With respect to *B. bigemina*, four different genotypes were found in Formosa and it was frequent to find three or even four different genotypes in the same sample. Conversely, in EEAM only two of the four known genotypes were detected and single infections appeared more often. The prevalence of all *B. bigemina* genotypes showed significant differences between the two epidemiological areas, but within Formosa significant differences also appeared for the M and P genotypes (Supplementary Table S1).

Regarding multiple infections of individual hosts by different agents, from a total of 157 positive samples, 82 (52.2%) had single infections. The number of species per sample was recorded and is shown in Fig. 4. Significant differences were observed between the two areas with and without tick control ($p < 0.0001$) but not between both tick-infested farms, LS and SM. As expected, the diversity of parasites per sample was increased in the area where ticks are not controlled. As an example, 25 bovines out of 38 from LS were infected with up to 4 different pathogens. Interestingly, *B. bovis* and *T. vivax* were never found as single infections. In order to detect associations between species, correlation coefficients were calculated with the Pearsońs method but results showed no evidence of significant association (Supplementary Tables S2 and S3).

With regard to *Trypanosoma* species, 72 (38.7%) samples were positive and, as mentioned above, the RLB assay also allowed the identification of a new variant or species of unknown origin. Comparison between T-PCR of Formosa samples and RLB showed that the latter was equal or better at identifying the correct species in 91.3% of the samples tested. Only in a small subset of 8 samples (8.7%) RLB failed to identify species that were found by PCR and sequencing.

### 3.3. Phylogenetic analysis of trypanosomes

Phylogenetic analysis was carried out using V7V8 PCR sequences from the trypanosome positive samples, which were aligned using ClustalW. Sequences of *T. theileri* and *T. vivax* from other American and African isolates were retrieved from GenBank and included in the analysis and *T. cruzi* was used as outgroup. Trees inferred by Maximum Likelihood (Fig. 5) and Neighbor Joining (not shown) displayed the same topology, branching in three major clusters. One cluster includes the *T. theileri* isolates, the second holds *T. vivax* isolates and a third one contains a different group of sequences which we provisionally decided to call "*T.* sp. Formosa" ($N = 11$). For *T. theileri*, samples from LS and SM clustered together and with other American isolates, separated from the African ones. In the case of *T. vivax*, sequences from Formosa formed two separate groups along with other American isolates, clearly separated from African strains. The third group of *T.* sp. Formosa sequences clustered in a separate group with a strong supporting bootstrap value and well differentiated from the other two species. All the sequences of *T. theileri* and *T.* sp. Formosa obtained in this study were identical among each other, regardless of which farm the sample was taken, meanwhile *T. vivax* sequences showed a certain degree of diversity among two subgroups (Fig. 5). This was also appreciated when values obtained from the distance matrix were analyzed (data not shown). *T. vivax* sequences present greater differences among each other (evolutionary distance of 0.011) than *T. theileri* and *T.* sp Formosa which showed minimal distances (0.001 and 0 respectively).

## 4. Discussion

Numerous vector-borne pathogens are a major constraint for livestock production in tropical and subtropical regions. For this reason, there is a need for more extensive tools to assess the prevalence of these pathogens and the possible association with each other in order to develop effective methods to control these diseases.

In this study, a RLB assay for detection of trypanosomes of veterinary relevance was developed and further combined with previous assays for *Babesia*, *Theileria*, *Anaplasma* and *Ehrlichia*. The new trypanosome probes developed here could be added to the existent RLB assays (Bekker et al., 2002; Gubbels et al., 1999) in order to have a single test for simultaneous detection and identification of all vector-borne pathogens that affect cattle. Based on bioinformatics analyses of the V7V8 hypervariable region of the trypanosome 18S rRNA gene, new primers and probes specific for these flagellates were designed and included in the new RLB assay. When tested, these oligonucleotides

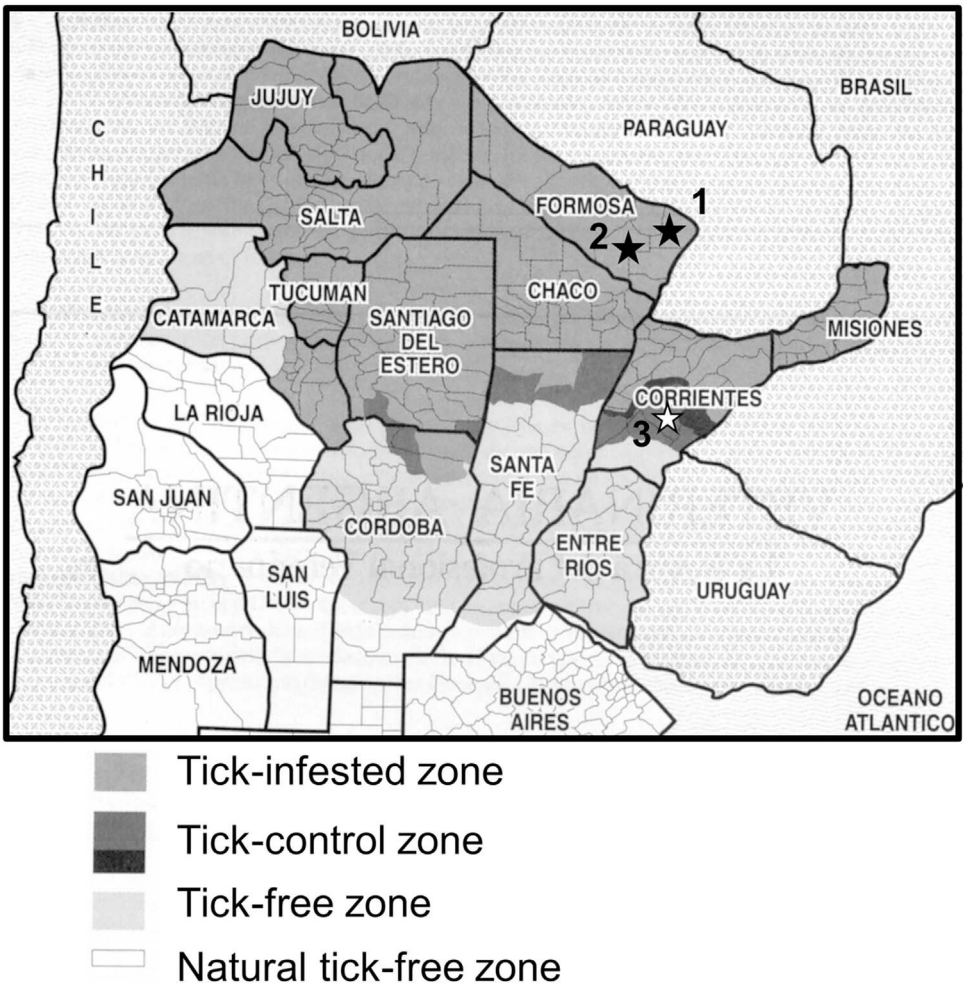



**Fig. 3.** Map of northern Argentina showing the location of the farms where animals were sampled. 1, La Salvación; 2, Santa María; 3, EEA Mercedes.

**Table 4**
Occurrence of different species identified in cattle blood samples using the RLB assay.

| | La Salvación (n = 38) | Santa María (n = 54) | EEA Mercedes (n = 94) | Total (n = 186) |
|---|---|---|---|---|
| *B. bovis* | 4 (10,5%) | 1 (1,9%) | 0 | 5 (2,7%) |
| *B. bigemina* | 8 (21,1%) | 11 (20,4%) | 55 (58,5%) | 74 (39,8%) |
| *B. bigemina genotype M* | 8 (21,1%) | 1 (1,9%) | 0 | 8 (4,3%) |
| *B. bigemina genotype P* | 7 (18,4%) | 2 (3,7%) | 0 | 9 (4,8%) |
| *B. bigemina genotype A* | 7 (18,4%) | 9 (16,7%) | 54 (57,4%) | 70 (37,6%) |
| *B. bigemina genotype T* | 6 (15,8%) | 6 (11,1%) | 29 (30,9%) | 41 (22%) |
| *A. marginale* | 27 (71,1%) | 25 (46,3%) | 39 (41,5%) | 91 (48,9%) |
| *A. centrale* | 11 (28,9%) | 9 (16,7%) | 0 | 20 (10,8%) |
| *T. evansi* | 0 | 0 | 0 | 0 |
| *T. theileri* | 7 (18,4%) | 25 (46,3%) | 4 (4,3%) | 36 (19,4%) |
| *T. vivax* | 2 (5,3%) | 13 (24,1%) | 1 (1,1%) | 16 (8,6%) |
| *T.* sp. Formosa | 11 (28,9%) | 5 (9,3%) | 0 | 16 (8,6%) |

were specific and sensitive enough for the effective detection of trypanosomes in all infected samples analyzed in this study. This result shows that the strategy based on the bioinformatic analysis of available sequences used for oligonucleotide design was successful, giving us the possibility of further extending the membrane to detect other species present in bovines or other hosts or vectors that may result of interest in the future.

For the first time in Argentina, data of the prevalence of bovine trypanosomes were obtained through molecular methods such as the RLB developed here. Previous reports showed microscopic observation of bovine trypanosomatids in blood smears but the species identification was done solely based on morphological measurements using light microscopy (Monzón et al., 2010, 1995). With our results we can unequivocally say that the species *T. theileri* and *T. vivax* are circulating with high prevalence in the province of Formosa. Although there are previous reports of the detection of *T. evansi* in cattle from Asia, Europe and Africa (Fereig et al., 2017; Sivajothi et al., 2016; Takeet et al., 2013; Weerasooriya et al., 2016), this parasite was never detected in our samples. One possible explanation could be that, the breeds of the region studied here are not susceptible to the *T. evansi* infection.

Notably, the prevalence of *T. theileri*, a non-pathogenic trypanosome, was higher than *T. vivax* which causes severe disease. This could be explained by the possibility that *T. theileri*, unlike *T. vivax*, is transmitted not only by biting flies (Desquesnes, 2005), but also by ticks (Burgdorfer et al., 1973; Latif et al., 2004; Martins et al., 2008; Morzaria et al., 1986; Shastri and Deshpande, 1981). Further studies should be done in order to determine which tick vector could be transmitting *T. theileri* in our region and the prevalence of both *T. theileri* and *T. vivax* in bordering provinces with similar agro-ecological conditions to Formosa.

The RLB hybridization assay developed here not only works as a detection tool but also has a prospective power. When a sample is positive only for a genus-specific probe, further sequencing of the PCR products permits the identification of sequence polymorphisms that could lead to the identification of new species or genotypes. This analysis could lead to the re-design of the species-specific probes to detect the new genotype or species. In previous results of our group, this was

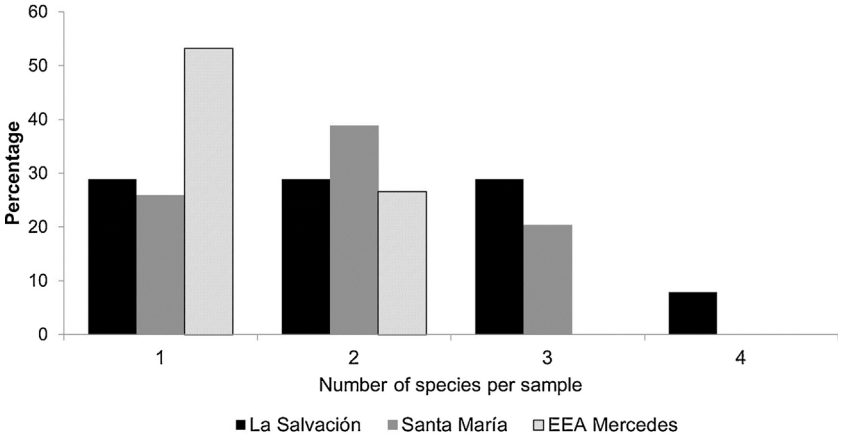



**Fig. 4.** Analysis of field samples by reverse line blot hybridization. Number of species found per sample in the farms studied.

found for *B. bigemina* in which the species probe of the commercial membrane did not hybridize with local isolates (Petrigh et al., 2008). In this study, the prospective potential of the RLB allowed the identification of a group of samples bearing an unknown bovine trypanosome that has not yet been characterized in detail. Additional analysis of the complete 18S rRNA and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes are the subject of future studies as described by Lima et al., 2013 for bat trypanosomes. These studies will allow determining if *T.* sp. Formosa is effectively a new species of bovine trypanosome.

Phylogenetic analysis of trypanosomes showed that all the *T. theileri* found in Argentina are identical and form a single group with other American isolates. On the other hand, *T. vivax* clustered in two groups, also showing more identity with American isolates than with the African ones. As previously mentioned, the group of sequences that could not be attributed to any trypanosome previously described and named provisionally by us as "*T.* sp. Formosa", clustered together in a separate tree branch with maximal supporting bootstrap value. Similarly to the case of *T. theileri*, all sequences from this new potential species are identical even though they come from two different farms in the province of Formosa, separated by more than 100 km.

In this study, the prevalence of vector-borne parasites was assessed in two epidemiologically different areas. In general, in areas with tick control, animals are not vaccinated and showed lesser infection rates in all parasitic species with the exception of *B. bigemina.* The differences found here could be attributed to the fact that *B. bovis* infected cattle usually have lesser circulating parasites in peripheral blood because of cytoadherence of the parasite to peripheral capillaries (Callow and McGavin, 1963; O'Connor et al., 1999) which could account for the lower sensitivity of the RLB technique for this species. *A. centrale* was absent in EEAM because in this farm the vaccine against anaplasmosis is not currently used and in Argentina there is no evidence of the natural circulation of *A. centrale* (Torioni de Echaide et al., 2008).

Within the area without tick control, there were no significant differences between the two farms sampled (LS and SM) in the prevalence of both *Babesia* species, *A. centrale* and *T. theileri*. However, significant differences were observed for *A. marginale*, *T. vivax* and *T.* sp. Formosa. These differences could be due to various factors that may influence the prevalence of parasites, such as vector density, abundance of cattle, age of the host and resistance of the host to the parasites or their vectors (Eygelaar et al., 2015). These variables deserve additional studies and are beyond the scope of this work.

Regarding coinfections, there was more diversity of parasites per sample in farms from Formosa than in EEAM which is located in the province of Corrientes. This is consistent with the absence of tick management practices in the former province. It is worth mentioning that after correlation analysis with Pearson's method, there was no evidence of significant association between any of the analyzed species, which may suggest that neither competition nor facilitation, are acting between the detected species.

It should be taken into account that the RLB design used in this study was based on the most prevalent vector-borne pathogens present in Argentina. However, the detection potential of the membrane can be easily expanded for other vector borne pathogens present in bovines (in blood or other tissues) or even in the vectors from this and other geographical regions (Abdallah et al., 2017).

In conclusion, the RLB optimized in this work is a molecular detection tool that allows the simultaneous identification of eight species of bovine hemoparasites from different genera. The membrane also permits the identification of coinfections in a large number of samples at the same time, which makes this assay an essential tool for epidemiological survey. The RLB developed here has also a prospective power since we were able to provide molecular evidence of the presence of trypanosomes in northern Argentina of known and unknown species. Finally, this study also illustrates the diversity of vector-borne parasites that affect cattle in northeast Argentina and provides a valuable tool that improves our capacity for integrated epidemiological survey.

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## Ethical statement

All the blood samples used in this work were obtained by cattle manually restrained (< 5 min) and were aseptically collected by jugular venipuncture (Vacutainer ™, Becton Dickinson, < 0.0005% blood volume; one sampling per animal). The protocol for animal handling and venipuncture was performed following the guidelines of the Institutional Committee for the Use and Care of Experimentation Animals (protocol approval No. 025/2011). All samples came from privately owned herds and were sampled with the approval of the owners.

## Conflict of interest statement

All the authors have participated in the study and no conflicts of interests have been disclosed.

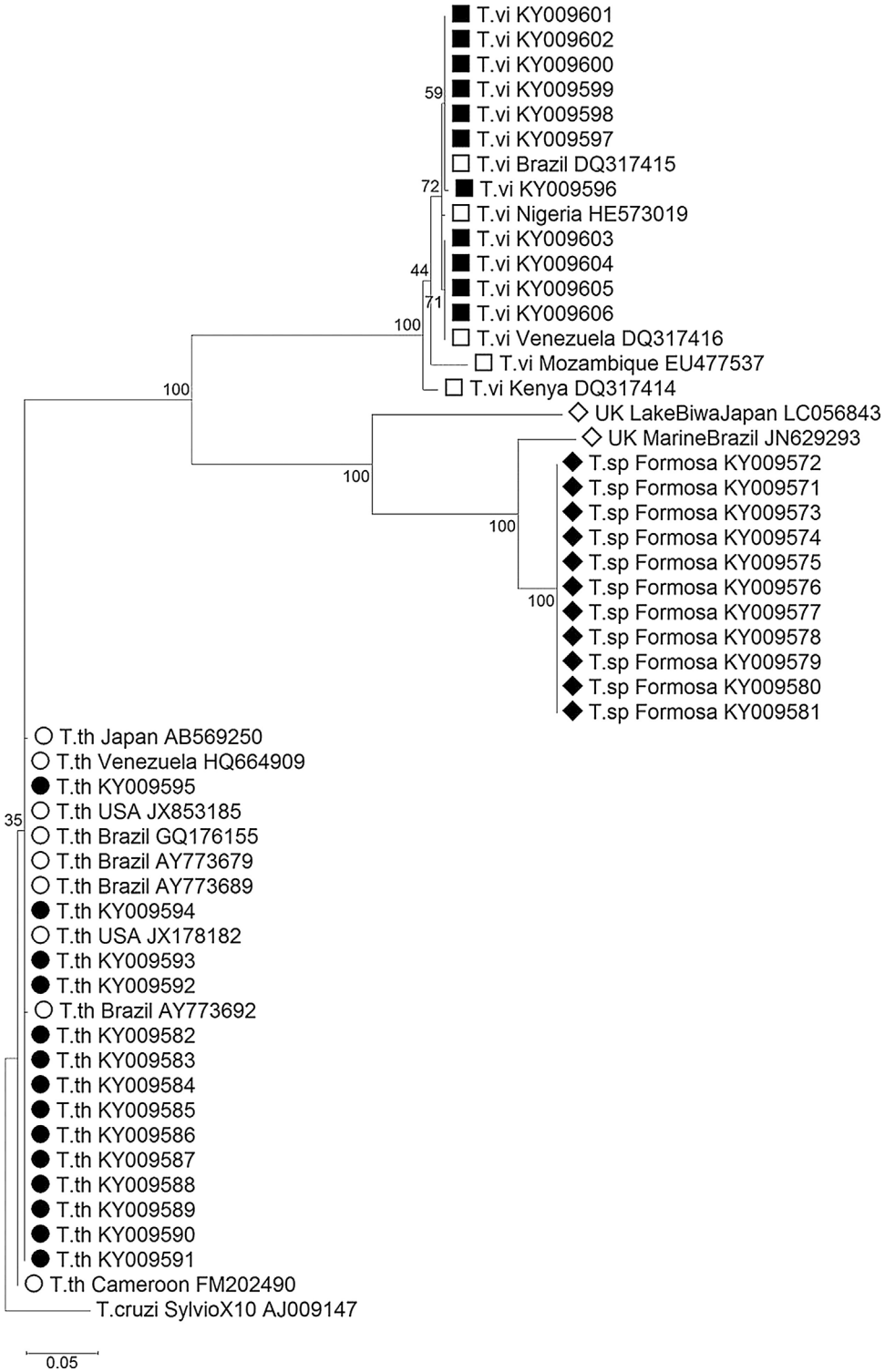


**Fig. 5.** Molecular phylogenetic analysis inferred by Maximum Likelihood method. A fragment of 463 bp of the V7V8 hypervariable region of the 18S rRNA gene of different *Trypanosoma* species was used for the analysis. Bootstrap values are shown next to branches. Filled circles, squares and diamonds correspond to sequences obtained in this study, while empty figures indicate sequences retrieved from the Genbank database.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ttbdis.2017.08.011.

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