Research Article

Molecular Detection of Theileria spp. in Livestock on Five Caribbean Islands

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Theileria spp. are tick-transmitted, intracellular apicomplexan protozoan parasites infecting a wide range of animals. As there is very limited information on the prevalence of Theileria spp. in the Caribbean we used the recently described genus-specific pan-\textit{Theileria} FRET-qPCR to identify infected animals in the region and a standard 18S rRNA gene PCR and sequencing to determine the species involved. We found \textit{Theileria} spp. in 9% of the convenience samples of animals (\(n = 752\)) studied from five Caribbean islands. Donkeys (20.0%; 5/25) were most commonly infected, followed by sheep (17.4%, 25/144), cattle (6.8%; 22/325), goats (5.0%; 12/238), and horses (5.0%; 1/20). Six species of \textit{Theileria} were identified: \textit{T. equi} (donkeys, cattle, goats, and sheep), \textit{Theileria} sp. OT3 (sheep and goats), \textit{Theileria} sp. NG-2013a (cattle), \textit{Theileria} sp. YW-2014 (donkeys), \textit{Theileria} sp. BI5a (goats), and \textit{Babesia vulpes} or a closely related organism (sheep and goats). Only \textit{T. equi} has been previously reported in the Caribbean. Our findings expand the known host ranges of \textit{Theileria} spp. and the known distribution of the organisms around the world.

1. Background

\textit{Theileria} spp. are tick-transmitted, intracellular apicomplexan protozoan parasites infecting leukocytes and erythrocytes of a wide range of animals [1, 2]. The organisms have been described in all livestock species and can cause significant economic losses to farmers. They are transmitted by a variety of ixodid ticks of the genera \textit{Rhipicephalus}, \textit{Hyalomma}, \textit{Amblyomma}, and \textit{Haemaphysalis} [3]. Infections with some \textit{Theileria} spp. can result in fever, anemia, hemoglobinuria, and death in severe cases, but many species are benign and cause minor or no signs. Animals that recover from acute or primary infections usually remain persistently infected and may act as reservoirs for tick vectors [4, 5]. Infected animals are found particularly in tropical and subtropical regions in Africa, the Middle East, Southern Europe, and Asia [6–11].

There is little information on infectious agents in livestock in the Caribbean although animal production is an important source of income for many people in the region. In the case of \textit{Theileria} spp., morphological and serological evidence has been presented that \textit{T. mutans} and \textit{T. velifera}, both benign species transmitted by \textit{Amblyomma} spp., occur in cattle on Guadeloupe [12]. Also, an organism with the morphology of \textit{T. mutans} was seen in a blood smear from a bovine on Martinique [13]. In Trinidad, \textit{T. equi} (previously \textit{Babesia equi}) has been demonstrated in horses with a specific nested 18S rRNA PCR [14, 15] and a serosurvey has provided supporting evidence for its presence [16].

While there are many tests to detect \textit{Theileria} spp. in animals, their specificity varies, as does their usefulness in finding the full spectrum of organisms present in an area. Microscopic detection of parasites can be difficult with low parasitemia and does not readily allow differentiation of species [2]. Serological studies, although sensitive and relatively easy to perform, are not specific as there is cross-reactivity between \textit{Theileria} spp. [17]. Although molecular techniques have been described, many are for specific species which limits their usefulness in surveys. Reverse line blotting (RLB) assays enable the simultaneous identification of multiple species [18, 19], but they are cumbersome and time demanding to perform and identifying stringent...
species-specific oligonucleotide sequences can be challenging [20]. Recently, a genus-specific, species-specific pan-Theileria FRET-qPCR has been described that detects the recognized Theileria spp. of domestic animals in a single reaction (Table 1) [21]. To provide further data on Theileria spp. in the Caribbean, we used the pan-Theileria FRET-qPCR to screen livestock from five islands for evidence of infection. Further, we used a standard 18S rRNA PCR and gene sequencing on positive reactors to identify the Theileria spp. involved. The results of this survey are described below.

2. Materials and Methods

2.1. Samples Collection. Jugular venipuncture was used to collect blood in EDTA from convenience samples of apparently healthy livestock (cattle, goats, sheep, donkeys, and horses) on five Caribbean islands [22]. This study was reviewed and approved by the Institutional Animal Care and Use Committee of the Ross University School of Veterinary Medicine (RUSVM), St. Kitts. Owners of animals gave permission for the blood samples to be collected.

2.2. DNA Extraction. The DNA was extracted from aliquots (200 μL) of the whole blood samples with the QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. The DNAs were eluted into 200 μL Buffer AE and couriered to Yangzhou University College of Veterinary Medicine, China, at room temperature where they were frozen at −80°C until PCRs were performed.

2.3. PCRs for Theileria Detection and Species Determination. All the PCRs were performed on a Roche Light-Cycler 480-II platform with the HMBS gene as an endogenous control [23]. Samples found positive in the pan-Theileria FRET-qPCR were tested in a conventional PCR with primers targeting a highly polymorphic 584–610-nucleotide region of the 18S rRNA gene of Theileria spp. (Table 2, Figure 1) [21]. The amplicons from positive conventional PCRs were sequenced directly with forward and reverse primers to determine the Theileria spp. present (BGI, Shanghai, China) [21] as has been done with 18S rRNA sequences in a number of previous studies [11, 14, 17, 21, 24].

3. Results and Discussion

The sensitive and species-specific pan-Theileria FRET-qPCR [21] we used in our study demonstrated that a substantial proportion of livestock (8.6%; 65/752) on the five Caribbean islands we studied were infected with Theileria (Table 3). Each island had positive animals and each livestock species we studied was found to be infected with Theileria spp. Sequencing of representative samples of positive 18S rRNA PCR amplicons we obtained (n = 43) showed that there was one recognized Theileria spp. (T. equi) present in the Caribbean, along with five less well-characterized Theileria spp. (Tables 3 and 4, Figure 2). The average copy number of 18S rRNA per mL whole blood was relatively low at 116.6 ± 440.8, indicating that the animals we studied were chronically infected.

Theileria equi and the Theileria spp. YW-2014 were the species we identified in equids. Theileria sp. YW-2014 has been described in a Sika deer (Cervus nippon) from Japan but there is little sequence data on the organism with only a 552 bp sequence of the 18S rRNA gene reported in GenBank (AB981984). On the other hand, T. equi is a well-recognized cause of equine piroplasmosis [25], an important disease of horses which has been recognized and studied in the Caribbean [14–16]. The organism has also been found in dogs in Spain [26], South Africa [27], and Nigeria [28] with some having clinical signs that responded to appropriate treatment [29]. Our finding that T. equi also occurs in domestic ruminants further expands the host range of the organism. The significance, extent, and consequences of infections with T. equi in domestic ruminants require further investigation.
The table below shows the alignment of the nucleotides used in the primers and probes of the pan-*Theileria* FRET-qPCR used in this study.

| Forward primer | LCRed-640 | 6-FAM | Reverse primer |
|----------------|-----------|-------|----------------|
| **T. orientalis** | TAGTGACAAGAAATAA CAATACOGGGC | --TT | -- | |
| **T. buffeli** | GCTTTGTAATT GGAATGATGGAATT | A | AAACCTCTTCCAGATGTAATTGG | |
| **T. annulata** | -- | -- | AAACCTCTTCCAGATGTAATTGG | |
| **T. sergenti** | -- | -- | AAACCTCTTCCAGATGTAATTGG | |
| **T. luwenshuni** | -- | -- | AAACCTCTTCCAGATGTAATTGG | |
| **T. velfera** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **T. ovis** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **T. parva** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **T. tenuiberghi** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **T. lestoquardi** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **T. equi** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **T. separata** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **T. capreoli** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **T. bicornis** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **T. taurotragi** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **Theileria** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **sp. OT3** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **Theileria** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **sp. NG** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **Theileria** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **sp. B15** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **B. vulpes** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **B. hongkongensis** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **B. divergens** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **B. bovis** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **B. bigemina** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **B. gibsoni** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **B. microti** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **B. felis** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **B. canis** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **H. americanum** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **C. felis** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **T. gondii** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **C. T. AAT** | -- | -- | TGAACCTCCAGATGTAATTGG | |
| **G** | -- | -- | TGAACCTCCAGATGTAATTGG | |

Primers and probes are shown at the head of the table. Dots indicate nucleotides identical to primers and probes, and dashes indicate absence of the nucleotide. The upstream primer is used as the demonstrated sequences without gaps while the two probes and downstream primer are used as antisense oligonucleotides. The designed oligonucleotides show minimum mismatching with *Theileria* spp. The 6-FAM label is directly attached to the 3-terminal nucleotide of the fluorescein probe, and the LCRed-640 fluorescein label is added via a linker to the 5'-end of the LCRed-640 probe. The 18S rRNA sequences for the available recognized *Theileria* spp. on GenBank and other closely related protozoan species were obtained from GenBank: *T. orientalis* (HM388222), *T. buffeli* (HQ840967), *T. annulata* (KF429799), *T. sergenti* (EU083804), *T. luwenshuni* (JX469527), *T. velfera* (A097993), *T. ovis* (AY508458), *T. parva* (L02366), *T. tenuiberghi* (IF79835), *T. equi* (AY534482), *T. lestoquardi* (JQ974588), *T. separata* (AY26075), *T. capreoli* (AY726011), *T. bicornis* (AF499604), *T. taurotragi* (L19082), *B. vulpes* (JX454779), *Theileria* sp. OT3 (K61082), *B. hongkongensis* (JX454779), *Babesia* sp. (L02366), *Theileria* sp. B15 (JN572700), *B. hongkongensis* (L19082), *B. minuta* (L02366), *B. bigemina* (L02366), *B. gibsoni* (AF244912), *B. felis* (AF244912), *B. canis* (HM390440), *Hepatozoon americanum* (AF76468), *Cytauxzoon felis* (AY79805), and *Toxoplasma gondii* (L37415).
**Table 2: Primers and probes used in this study.**

| PCR          | Primer/probe | Nucleotides sequence                                                                 | Amplicon                  |
|--------------|--------------|--------------------------------------------------------------------------------------|---------------------------|
| FRET-qPCR    | Forward      | 5' - TTAGGCAAAGAATAACGTACCGGGTT - 3'                                                  | 178 bp                    |
|              | Reverse      | 5' - CAGCAGAAATTCATCACTACGAGCTTTTAACT - 3'                                           |                           |
|              | 6-FAM        | 5' - CAAATTGATACCTGGAAGGTTT-(6-FAM) - 3'                                             |                           |
|              | LCRed-640    | 5' - (LCRed640) - AATTTCCCATCATGCCAATTACAGAC - Phosphate - 3'                        | T. orientalis 593 bp; T. buffeli 591 bp; T. annulata 591 bp; T. sergenti 591 bp; T. luwenshumi 594 bp; T. velifera 592 bp; T. ovis 595 bp; T. parva 592 bp; T. uilenbergi 592 bp; T. equi 596 bp; T. cervi 595 bp; T. lestoquardi 591 bp; T. separata 593 bp; T. capreoli 599 bp; T. bicornis 610 bp; T. taurontragi 587 bp; T. mutans 585 bp; B. vulpes 609 bp; Theileria sp. OT3 600 bp; Theileria sp. NG 597 bp; Theileria sp. YW 593 bp; Theileria sp. B15 584 bp |
| Conventional | Upstream     | 5' - CCGAGGAGGGCTACACATCT - 3'                                                       |                           |
| PCR          | Downstream   | 5' - GAGCTACGACGCTATCTGACG - 3'                                                      |                           |
Table 3: Prevalence of *Theileria* spp. in livestock from five Caribbean islands.

|          | Bovine | Goat | Sheep | Donkey | Horse | Total | *Theileria* spp. |
|----------|--------|------|-------|--------|-------|-------|------------------|
| **Dominica** | 3/77 (3.9%) | 0/70 (0.0%) | 1/15 (6.7%) | N/A | N/A | 4/162 (2.5%) | *T. equi*, *Theileria* spp. NG-2013a, *B. vulpes*, or closely related organism |
| **Grenada** | N/A | 2/31 (6.5%) | N/A | N/A | N/A | 2/31 (6.5%) | *Theileria* spp. B15a |
| **Montserrat** | 0/12 (0.0%) | 8/19 (42.1%) | 24/62 (38.7%) | N/A | N/A | 32/93 (34.4%) | *Theileria* spp. OT3, *B. vulpes*, or closely related organism |
| **Nevis** | 19/43 (44.2%) | 2/114 (1.8%) | 0/41 (0.0%) | N/A | N/A | 21/198 (10.6%) | *T. equi*, *Theileria* spp. NG-2013a |
| **St. Kitts** | 0/193 (0.0%) | 0/4 (0.0%) | 0/26 (0.0%) | 5/25 (20.0%) | 1/20 (5.0%) | 6/268 (2.2%) | *T. equi*, *Theileria* spp. YW-2014 |
| **Total** | 22/325 (6.8%) | 12/238 (5.0%) | 25/144 (17.4%) | 5/25 (20.0%) | 1/20 (5.0%) | 65/752 (8.6%) | |

*No specimen was available.*

Table 4: The *Theileria* spp. identified in livestock from five Caribbean islands and their similarity with reported organisms on GenBank.

| *Theileria* spp. | Sequences identified in this study | Source | Highly similar sequences in GenBank | Mismatch |
|------------------|-----------------------------------|--------|------------------------------------|----------|
| *T. equi*        | 14                                | 8 cattle, 1 goat from Nevis; 1 cow from Dominica; 3 donkeys, 1 sheep from St. Kitts | KF559357 Horse from China | 0/550 |
| *Theileria* spp. OT3 | 11                                | 8 sheep, 3 goats from Montserrat | KF470868 Sheep from China | 0/555 |
| *Theileria* spp. NG-2013a | 7                                 | 6 cattle from Nevis; 1 cow from Dominica | KF597076 Waterbuck from Kenya | 0/552 |
| *Theileria* spp. YW-2014 | 1                                 | 1 donkey from St. Kitts | AB981984 Sika deer from Japan | 0/549 |
| *Theileria* spp. B15a | 1                                 | 1 goat from Grenada | JN572700 Buffalo from South Africa | 0/539 |
| *B. vulpes* or closely related organism | 9                                 | 4 sheep, 4 goats from Montserrat; 1 goat from Dominica | JX454779 Dog from France | 0/178 |

Numerous species of *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Ixodes*, and *Rhipicephalus* are confirmed or suspected vectors of *T. equi* [29]. Of these, only *A. cajennense* [30], *R. microplus* [30], *R. sanguineus* [31], and *R. turanicus* (unpublished observation) occur in the Caribbean. There is conflicting data that *Amblyomma cajennense* is a competent vector of *T. equi* [32] but the tick is localized to Jamaica, Trinidad, and Cuba in the Caribbean [30] and it appears, then, not to be the vector of *T. equi* we found on Nevis, St. Kitts, and Dominica. *Dermacentor nitens*, the tropical horse tick, is very common in the Caribbean and the tropical Americas [33]. Although there is no data on the competence of *D. nitens* as a vector of *T. equi* and there is contradictory epidemiological evidence [32], PCR positive *D. nitens* have been found [32] and Asgarali et al. [16] have suggested that this tick is a vector in Trinidad. They also suggested that *R. microplus* [16], which is very common on cattle throughout the Caribbean, might also be a vector. While there is some evidence that *R. microplus* is a competent vector [34] and our PCR identified *T. equi* in cattle on two islands, it seems unlikely that *R. microplus* is an important natural vector as it is a one host species and transovarial transmission has not been demonstrated [32]. Although *R. sanguineus* and *R. turanicus* have been implicated as vectors of *T. equi*, more recent studies have failed to confirm their role [29]. Further studies are needed to establish the epidemiology of *T. equi* and its vectors in the Caribbean and the neighboring Americas [32].

*Theileria* spp. OT3 was first described in sheep, deer, and chamois in Spain [35–37] and later in sheep in China [39], and Turkey [40]. Its pathogenicity and vectors have yet to be determined. Ours is the first report of the organism in the Caribbean and also the first report of the *Theileria* spp. OT3 in goats which further demonstrates that the organism has a wide distribution and host range. Although we used only convenience samples which were
African buffalo (Syncerus caffer) and it therefore appears that the pan-Theileria FRET-qPCR might not be as genus specific as first thought. Further work is currently underway in our laboratory to more clearly characterize the B. vulpes or closely related organism found in the Caribbean.

4. Conclusions

Our study has confirmed the sensitivity of the pan-Theileria PCR in the rapid detection of a wide range of Theileria spp. but has also shown it might detect B. vulpes or closely related
related organisms. We found livestock infected with *Theileria* spp. on each of the five islands we studied. While we could not confirm previous reports of *T. mutans* and *T. vivida* in cattle, we found that one recognized species, *T. equi*, four poorly characterized *Theileria* spp., and *B. vulpes* or a closely related organism are present in the region. Further studies are indicated to more precisely determine the phylogenetic relationships of these organisms in the Caribbean with closely related organisms from other parts of the world. Also, the prevalences of infections on the different islands should be determined as well as the impact these poorly characterized organisms might have on livestock production, both in the Caribbean and around the world where they are found.

**Conflict of Interests**

The authors declare that they have no competing interests.

**Authors’ Contribution**

Chengming Wang, Patrick Kelly, and Jilei Zhang designed the study, analyzed the data, and wrote the paper. Jilei Zhang, Jing Li, and Chuanling Xu carried out the experiments. All authors read and approved the final paper.

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