Theileria orientalis Ikeda Genotype: Implications for Cattle Health in Virginia

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Abstract

Of the four most economically important tickborne diseases of cattle in the world, two have been identified in Virginia, occasionally as co-infections: anaplasmosis and theileriosis. The latter is caused by the emerging infectious agent, the *Theileria orientalis* complex, in particular the Ikeda and Chitose genotypes. These organisms are carried by the ixodid tick, *Haemaphysalis longicornis*, recently identified in the United States. Our work has been focused on initially identifying the protozoal organisms, crafting assays to aid in the identification of these organisms in clinically affected animals, and briefly examining the rate of co-occurrence of theileriosis and anaplasmosis. This is important, as *Anaplasma marginale* - the most common etiologic agent of anaplasmosis in cattle in Virginia - is treatable with a safe, effective, FDA-approved compound, whereas there is no currently approved treatment for theileriosis. Finally, we seek to contextualize theilerosis as a cause of infectious bovine anemia (IBA) and its expected economic impact on the cattle industry in Virginia.

General Audience Abstract

Theileriosis is a disease that infects cattle, caused by the blood parasite, *Theileria orientalis*, specifically two distinct genotypes of *T. orientalis*, Ikeda and Chitose. Theileriosis is transmitted to cattle by the Asian longhorned tick, *Haemaphysalis longicornis*, which was recently identified in the United States. Globally, theileriosis is one of four major tickborne diseases of cattle with significant economic importance, so the discovery of this parasite in the state of Virginia is of special importance to the cattle industry in Virginia. My work has revolved around making the initial discovery of *T. orientalis* Ikeda in the United States, and developing tests for cattle producers and veterinarians to use to help diagnose theileriosis in sick animals. Another tickborne disease of cattle, anaplasmosis, is caused by a bacterium, *A. marginale*. These two organisms have different biology, are transmitted by different ticks, and are treated differently, but cause identical clinical disease in cattle. In addition to identifying *T. orientalis*, we have developed a single test that can determine if sick cattle have *T. orientalis* or *A. marginale* – this is important, because the antibiotic used to treat *A. marginale* does not work to treat *T. orientalis*. In fact, there is no treatment for *T. orientalis* available in the United States. In addition to developing diagnostic assays, I seek to put into pathobiological, ecological, and economic context the importance of theileriosis on the cattle industry in Virginia.

Dedication

First and foremost, this work is dedicated to my family, in particular my parents, Michael and Marey, who have never wavered in their support. It is because of them that I am the curious, confident, and capable woman that I am today. My siblings, Grace, John, and Stephen, have ensured - quite effectively - that my ego remains of manageable size; I owe them a lot for this (even if it doesn't always feel like I should).

To my mentors, of whom I am blessed to have many. In particular, Tom Cecere, Phil Sponenberg, Kevin Lahmers, and Tanya LeRoith, for their patience, their guidance, their well-timed advice, and for teaching me how to ask the right questions (and for forgiving me for asking them at the wrong time).

To my friends, but especially Jen, for their patience with my schedule, my exhaustion, and for constantly getting the short shrift of my time - it is entirely because of you that I have any friends at all.

To my students, who have asked pertinent questions and encouraged active and engaging dialogue on many subjects, and who keep kindled in me a true desire to teach.

Finally, and perhaps most importantly, to the producers and the veterinarians of Virginia, who have given so much of their time and their effort to this and many other projects, often at their own expense. My work revolves around your livelihood, and it is my most sincere hope that for every inconvenience that my work has caused, I have contributed meaningfully to providing useful tools and information to serve your industry.

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Table of Contents

List of Figures

Introduction

Chapter 1

Chapter 3

List of Tables

Introduction

Chapter 1

Chapter 2

List of abbreviations

Ct: cycle threshold

ELISA: enzyme-linked immunosorbent assay

FDA: Food and Drug Administration

IBA: Infectious bovine anemia

ID: identification

mpsp: major piroplasm surface protein

NA: not available

NVSL: National Veterinary Services Laboratories

PCR: polymerase chain reaction

PCV: packed cell volume

ssu: small ribosomal subunit

VDACS: Virginia Department of Agriculture and Consumer Services

VDOC: Virginia Department of Corrections

ViTALS: Virginia Tech Animal Laboratory Services

USDA: United States Department of Agriculture

USDA-ARS-ADRU: U.S. Department of Agriculture, Agricultural Research Service, Animal Disease Research Unit

WADDL: Washington Animal Disease Diagnostic Laboratory

Introduction

Infectious bovine anemia is a clinical syndrome of growing concern in the United States, particularly in Virginia. The clinical syndrome is typified by anemia with icterus, weakness/unthriftiness, poor production, and abortions. The constellation of clinical signs contributes to decreased production of beef and dairy cattle, and globally is associated with substantial economic loss by this industry (Watts et al. 2016, Lawrence 2019). In Virginia in particular, there are two etiologic agents of concern that contribute to this disease, both tickborne diseases in cattle. In fact, among tickborne diseases of cattle, anaplasmosis and theileriosis are two of the four organisms with global economic importance; the other two are babesiosis and cowdriosis (ehrlichiosis)(Gebrekidan et al. 2020).

Anaplasma marginale is a rickettsial bacterium that infects red blood cells, transmitted by multiple tick species (Wickwire et al. 1987). As a bacterial agent, anaplasmosis is treatable; oxytetracycline is a safe and effective drug, and is approved for use in beef and dairy cattle in the United States.

Theileriosis is another tickborne disease of global economic importance (Gebrekidan et al. 2020). Included within this category are the lymphoproliferative, transforming diseases *Theileria parva* and *Theileria annulata*. Other theilerial organisms have historically been considered benign, incidental findings in cattle. However, there is increasing interest in the pathogenesis of a particular species complex, *Theileria orientalis*, the main subject of this dissertation.

The *Theileria orientalis* complex

Although the nomenclature of *T. orientalis* is yet unresolved, a recent study proposed classifying this organism as a single-species complex composed of multiple genotypes (Uilenberg 2011, Gebrekidan et al, 2020). Different sequencing schema based on different molecular markers categorize the complex differently, but genotyping according to the major piroplasm surface protein (mpsp) is increasingly common, in large part due to the mpsp's role as an antigenic target. According to this scheme, there are 11 genotypes within the *T. orientalis* complex: Chitose (type 1), Ikeda (type 2), Buffeli (type 3), types 4-8, N1, N2, and N3 (Sivakumar et al. 2014).

Ribosomal subunits (18S rRNA) are commonly used as sequencing targets to identify organisms of interest, in addition to crafting phylogenetic trees and exploring evolutionary biology of target organisms (Ng et al. 2018). Although this gene product is not involved in the pathogenesis of disease, the *ssu* gene is conserved across metabolic states and is constitutively expressed, making it a reliable target for organism identification based on sequence. There are 8 genotypes according to this scheme (Kakuda et al. 1998, Sivakumar et al. 2014).

There are benefits to categorizing the complex using either scheme, however, because our laboratory focuses on disease dynamics and pathogenesis, we utilize the *mpsp* schema more frequently. Additional molecular markers include internal transcribed spacers 1 and 2 of nuclear ribosomal DNA, a portion of the cytochrome c oxidase, and p23 (Gebrekidan et al. 2020).

All members of the *T. orientalis* complex are transmitted by *Haemaphysalis longicornis*, the Asian longhorned tick, an exotic tick species recently introduced to the United States (Beard et al. 2018, Rainey et al. 2018). As a protozoal agent, and in contrast to IBA induced by *A. marginale*, there are no approved drugs for treatment in food animals in the US for infected cattle.

Within the group, Chitose and Ikeda are both capable of causing disease alone or in conjunction (Kim et al. 2017, Lawrence et al. 2016, Lawrence et al. 2019, McFadden et al., Oakes et al. 2019, Watts et al. 2016). Buffeli is largely considered a benign, non-pathogenic constituent of the complex (Sivakumar et al. 2014, Watts et al. 2016). Interestingly, there is evidence to suggest that Buffeli is carried by ticks other than *H. longicornis* (Stewart et al. 1987). The tick vector for Buffeli in the United States is unknown, but there is evidence that this member of the complex has been in the US for some time (Stockham et al. 2000).

As a protozoal organism, *T. orientalis* has a two-host life cycle, in which the intermediate host is a tick and the final host is bovine. In the United States and elsewhere in the world, *H. longicornis* is the tick host for most members of the *T. orientalis* complex, except for Buffeli, as mentioned earlier. Although *H. longicornis* will infest a variety of mammalian species, *T. orientalis* typically proliferates in the blood cells of cattle. Within the bovine host, the protozoan subsists in red blood cells as a piroplasm, and in white

blood cells as sporozoites. The full life cycle is elucidated in detail below, and understanding the life cycle is crucial to understanding the pathogenesis of disease.

The protozoal life cycle

As with many protozoal organisms, *T. orientalis* exhibits both sexual and asexual reproduction. Sexual reproduction occurs in the gut of the tick host. Erythrocytes ingested by the tick from an infected bovid are lysed within the gut lumen, liberating *T. orientalis* piroplasms. These piroplasms differentiate into gametocytes, and undergo syngamy (fertilization). Resulting zygotes invade intestinal epithelium, where they undergo meiotic division and differentiate into kinetes. When the tick molts, kinetes migrate through the basal portion of the enterocytes into the hemocoel and to the salivary glands. In the salivary glands, the kinetes undergo sporogony and develop into multinucleated sporonts. When the tick feeds on a mammalian host, sporonts develop into sporozoites and are released into the blood of the mammalian host. In cattle, the sporozoites initially invade leukocytes and develop into schizonts. In some theilerial protozoan organisms, the leukocyte stage is critical to the pathogenesis; as previously mentioned, *T. parva* and *T. annulata* induce lymphoid proliferation, which contributes to severe disease in infected animals. This is the stage of the life cycle in which that occurs. In contrast, the leukocyte stage of *T. orientalis* is benign and transient; in fact, infected white blood cells are rarely seen in peripheral blood at all (Watts et al. 2016). The most pathogenically important life cycle stage in *T. orientalis* involves the red blood cells. After infecting leukocytes, schizonts reproduce asexually, lyse the white blood cells, and then infect erythrocytes, where they differentiate into piroplasms. The exact mechanism by which these piroplasms damage the red blood cells is not fully understood; piroplasm load is not always correlated with degree of anemia, suggesting that extravascular mechanisms contribute to anemia in addition to direct damage induced by piroplasms themselves (Kim et al. 2017, Lawrence et al. 2019). Potential molecular mechanisms of red blood cell damage are discussed later in this introduction.

Further, although there is work yet to be done in order to elucidate the factors of parasite persistence in bovid hosts, there is evidence that *T. orientalis* infection is chronic, perhaps for life, and that recrudescence of clinical disease occurs sporadically (Lawrence et al. 2019) or in the face of stress (Watts et al. 2016).

The tick

As with any two-host parasite, understanding the ecology of the intermediate host is important for contextualizing the epidemiology of the disease. In the case of *T. orientalis*, the intermediate host - the vector - is the ixodid tick *H. longicornis*, the Asian longhorned tick. This tick was first described in the United States in 2017 (Rainey et al. 2018) and has since been identified in Arkansas, Connecticut, Delaware, Georgia, Kentucky, Maryland, Missouri, New Jersey, New York, North Carolina, Ohio, Pennsylvania, Rhode Island, South Carolina, Tennessee, Virginia, and West Virginia (USDA 2021, Beard et al. 2018). The initial portal of entry into the country is unknown at this time, however, *H. longicornis* has been intercepted from imported animals during quarantine prior to the 2017 discovery (Beard et al. 2018).

Table 1. Distribution of *Haemaphysalis longicornis* by host and species – nine states, August 2017-September 2018. From Beard et al. 2018, used with permission.

Host category, no. (% of total)/Species	No. (% of host category)
Domestic animal, 23 (61)	
Cat	1 (4)
Cow	4 (17)
Dog	12 (52)
Goat	2 (9)
Horse	2 (9)
Sheep	2 (9)
Total	**23 (100)**
Wildlife, 13 (34)	
Coyote	1 (8)
White-tailed deer	7 (54)
Gray fox	1 (8)
Groundhog	1 (8)
Virginia opossum	2 (15)
Raccoon	1 (8)
Total	**13 (100)**
Human, 2 (5)	2 (100)
Total	**38 (100)**

Because *T. orientalis* relies on a vector for its pathogenesis, the distribution of theileriosis is restricted by the distribution of competent vectors. Unfortunately, the biology of the tick itself is problematic, as they are indiscriminate feeders, and will feed on multiple mammalian species; in the US, it has been described infesting both domestic and wild animals, from cats to groundhogs, and several cases of *H. longicornis* parasitizing human beings have also been reported (**Table 1**).

Further, this tick species is bisexual and capable of reproducing via parthenogenesis, complicating control of this vector species (Heath et al. 2016, Marendy et al 2020). Parthenogenesis is a form of clonal reproduction

in which females reproduce without the assistance of a male. Although the bisexual ticks and those capable of reproducing parthenogenically are karyotypically distinct (Marendy et al. 2020), the implications of this are multiple. Because a male is not needed for colony establishment, this tick will be capable of becoming endemic far more rapidly than non-parthenogenetic species. Additionally, male sterilization methods used to control other arthropod vectors may not be as successful in this tick species. In addition, the studies into seasonal activity of *H. longicornis* in the US indicate that nymphal ticks persist year round (Thompson et al. 2021).

In other parts of the world, *H. longicornis* is a vector for diseases of concern to humans, including the etiologic agent for human hemorrhagic fever, Dabie bandavirus (syn. severe fever with thrombocytopenia syndrome virus), the rickettsial species that causes Japanese spotted fever, and various species of *Anaplasma, Babesia, Borrelia,* and *Ehrlichia*, which contain potentially zoonotic species (Beard et al. 2018). No such diseases have yet been identified in *H. longicornis* ticks in the US, however, there are species of *Rickettsia, Borrelia, Ehrlichia, Anaplasma,* and viral agents that are endemic zoonoses in the United States, for which there is concern this tick may become a vector. In addition to the propensity to carry other infectious organisms, *H. longicornis* is hyperendemic, exhibiting so-called “swarming” behavior, wherein thousands of ticks can be found parasitizing a single host. Because they feed on blood, the high number of ticks alone can cause severe anemia.

Several tick modeling studies have examined *H. longicornis*’ predicted spatial distribution throughout North America (Rochlin 2019, Raghavan 2020). By comparing ecological niche factors of known international habitats of the tick to similar environments within United States, and applying robust statistical predictive methods, Raghavan et al (Raghavan et al 2019; permission granted figures 2 and 4) suggested areas where the tick was mostly likely to survive.

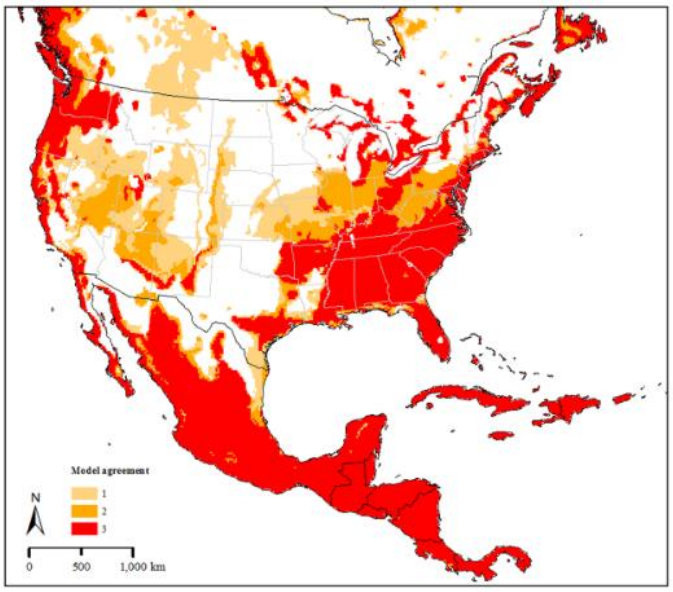

Figure 1. Predicted suitable areas for *Haemaphysalis longicornis* across North America. 1, 2, and 3 represent areas that were predicted to be suitable for the establishment of *H. longicornis* by one, two and three models, respectively. Darker areas represent progressively higher agreement between the models. From Raghavan et al. 2019. Used in accordance with the Creative Commons license: http://creativecommons.org/licenses/by/4.0/

This study concluded that *H. longicornis* has a large predicted range of geographical suitability, encompassing the southeastern United States with a focus of suitability within the pacific northwest and extending southward along the coast. A similar study conducted by Rochlin (2019) examining temperature, precipitation, and distribution throughout Australia similarly concluded *H. longicornis* is likely to persist along the southeastern coast of the United States and along parts of the western coast, as well.

The dual threat of *H. longicornis* and *T. orientalis* Ikeda was classified as one of the 15 most important biological conservation issues of 2020 (Sutherland 2020).

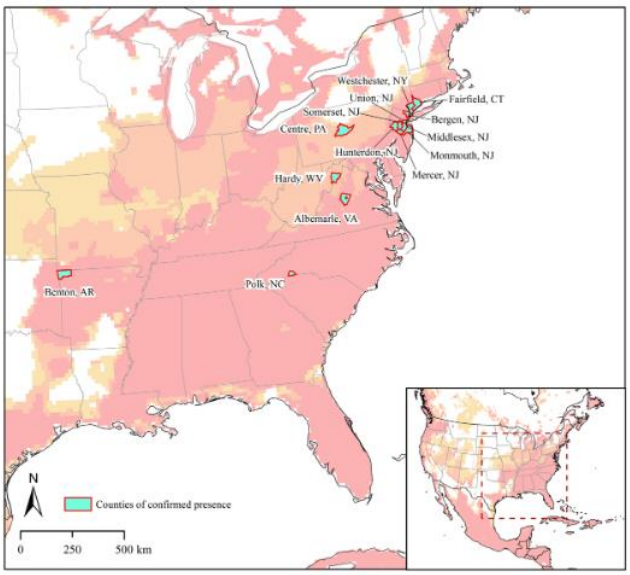

Figure 2. Counties in the United States in which the *Haemaphysalis longicornis* tick has been positively confirmed as of September 2018. Colors in the background show areas that are predicted suitable for the establishment of *H. longicornis* in North America; darker shades indicate higher degree of agreement among models. From Raghavan et al. 2019. Used in accordance with the Creative Commons license: http://creativecommons.org/licenses/by/4.0/

During the initial outbreak of *T. orientalis* Ikeda in Virginia, *H. longicornis* ticks were collected off of cattle within the index herd (Oakes et al 2019). Due to the presence of a known vector species of Ikeda, as well as clinical theileriosis, *H. longicornis* was thought to be the transmitter of disease in that herd. Since then, *T. orientalis* Ikeda has been recovered from *H. longicornis* ticks collected from the index farm (Thompson et al 2020) and subsequent transmission studies carried out by our collaborators have demonstrated that this tick is capable of transmitting *T. orientalis* in the United States

(Dinkel et al. 2021). Similar studies in Australia have likewise confirmed *H. longicornis* as a vector for *T. orientalis* (Marendy et al 2020).

Pathogenesis of Disease

Unlike other theilerial organisms, the disease caused by the pathogenic members of the *T. orientalis* complex tends to be characterized by poor-doing related to anemia, and is rarely fatal unless complicated by a second infection. Clinical signs include general malaise, anemia, and icterus, but abortion and occasionally sudden death are also reported (Watts et al. 2016, Yam et al 2018). The index case described in the United States is unique in that it appeared as an acute mortality event involving multiple animals (Oakes et al. 2019). This organism has recently been identified in New Zealand and Australia, and in the intervening years has become the most prominent etiology of bovine anemia in New Zealand (Lawrence 2019). Lessons learned from these two countries will be paramount in predicting and controlling the disease here.
Historically considered non-pathogenic, these two genotypes of the *T. orientalis* complex (Chitose and Ikeda) have been identified in cattle with clinical signs of fever, icterus, anemia, and chronic ill-thrift; rarely, disease is fatal, and in some cases it can cause abortions (McFadden et al. 2011, Oakes et al. 2019, Gebrekidan et al. 2020). Even cattle that are not anemic and who do not abort generally produce at lower rates than their herd mates (Lawrence et al. 2016, Lawrence et al. 2018). Chronic ill-thrift in these animals corresponds to decreased market weight of beef cattle, and a decrease in both quantity and quality of milk in dairy cattle. A report from Australia associated Ikeda with an annual loss of revenue of approximately 20 million Australian dollars (Lawrence et al. 2018, Yam et al. 2018), and another from New Zealand reports an annual loss of approximately 1 million New Zealand dollars by a single farm (Yam et al. 2018). These factors have yet to be fully investigated in the United States.
The mpsp of *T. orientalis* is expressed as a surface protein in the intraerythrocytic piroplasm stage of growth. Previous studies have suggested that the presence of the mpsp protein alone is associated with anemia, thought to be due to hemolysis (Kim, et al, 2017). In addition, different genotypes of the *mpsp*

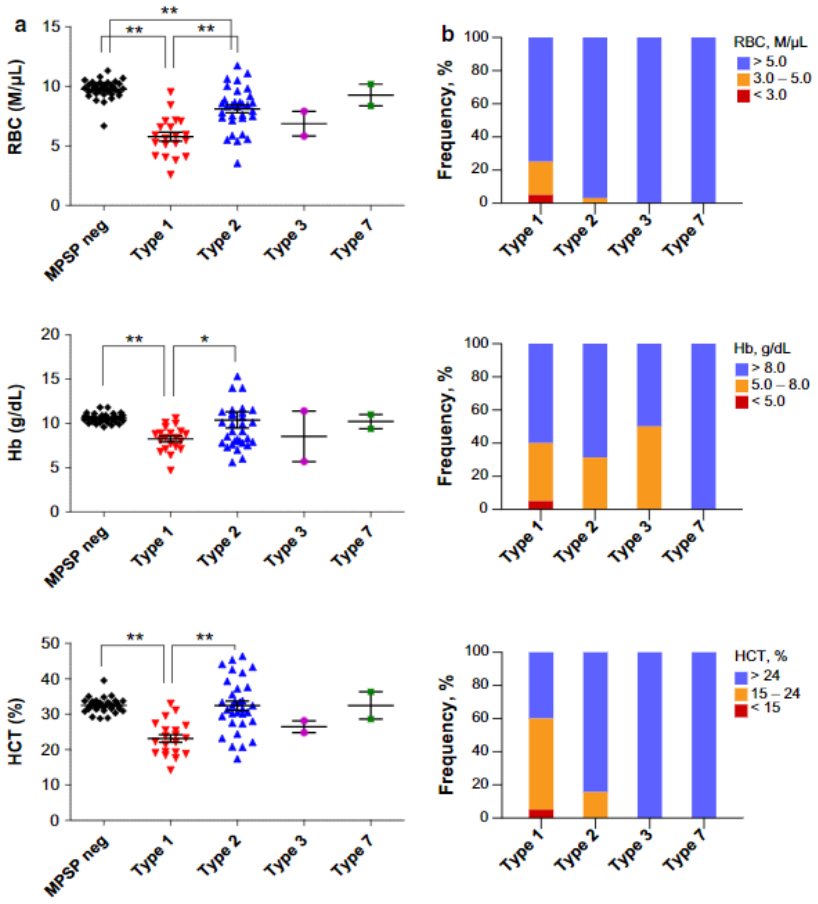

Figure 3. Development of anemia according to mpsp genotypes in *Theileria*-infected cattle. Comparison of red blood cell count (RBC), hemoglobin (Hb), and hematocrit (HCT) values among mpsp types (**a**) and the presence of anemia in each mpsp type (**b**). *Colored bars* indicate the level of anemia. *Blue* no anemia; *orange* mild anemia and *red* severe anemia. Number of animals: mpsp-negative (n=34); types 1 (n=20), 2 (n=32), 3(n=2) and 7 (n=2). *P<0.05 and **P<0.0001. From Kim et al. 2017. Used in accordance with the Creative Commons Attribution 4.0 International License: *http://creativecommons.org/publicdomain/zero/1.0/*

schema have been associated with anemia of varying severity, with one study specifically elucidating Chitose as inducing the greater decrease in red blood cell, hemoglobin, and hematocrit values compared to Ikeda or *mpsp*-negative samples (Kim, et al, 2017).

Disease ecology and context in Virginia

H. longicornis was first described in Virginia in 2019, in Albemarle county in Virginia's Piedmont region. This particular region of the state is responsible for much of the state's overall cattle production, so the discovery of *H. longicornis* and *T. orientalis* Ikeda in this area is concerning for cattle producers in this area and beyond. Since that initial discovery, *T. orientalis* Ikeda has additionally been found in Augusta county. Augusta has the 2nd largest number of beef cattle in the state; its neighbor, Rockingham county, houses the largest number. Consequently, an untreatable, chronic disease impacting beef production in this area of the state is deeply concerning for the producers in this area.

An epidemiologic study out of New Zealand demonstrates that calves are at increased risk of disease with *T. orientalis* than adults, and that beef calves specifically have a higher risk than dairy calves (Lawrence 2016). A tentative explanation for this difference between beef and dairy calves relates to their management, in that

dairy calves are typically kept in more sheltered environments than are beef calves. This trend apparently reverses when the animals are adults, with significantly more dairy cattle exhibiting anemia by the end of the study than beef calves (Lawrence 2016). The epidemiology of calf involvement is applicable to Virginia, as its primary cattle industry is beef cow-calf operations; therefore, the population of susceptible beef calves in Virginia is likely to be high.

Because *T. orientalis* is untreatable, and because clinical signs mimic those of treatable causes of bovine infectious anemia, rapid identification of the organism is critical for clinical and production decision-making. In addition, as mentioned earlier in this manuscript, *A. marginale* causes identical disease in affected animals. The focus of my doctorate work has largely been on describing *T. orientalis* and so the focus of this dissertation is the same, however, it is worth mentioning the rickettsial organism in brief.

Anaplasma marginale

This is a rickettsial bacterium and an intracellular parasite in the red blood cells of infected cattle. It is transmitted by numerous ticks endemic to Virginia, but is not transmissible via *H. longicornis* (Conell, 1978). Thus, the areas where *T. orientalis* complex and *A. marginale* co-occur rely on overlap of the geographic distribution of their respective tick vectors. Cattle described in the initial outbreak of Ikeda were found to be co-infected with *A. marginale* (Oakes et al. 2019).

Due to the presence of two distinct etiologic agents of IBA in Virginia, and because *T. orientalis* does not have an approved treatment in the US, its presence in this state is of concern to the cattle industry here. Our position as a diagnostic laboratory attached to an academic institution puts us in a unique position to develop, validate, and deploy assays to aid producers and veterinarians across the state, and it is our goal to do such. Further, the predicted distribution of *H. longicornis* and the ecologic difficulties presented by this parthenogenic tick suggests that theileriosis and anaplasmosis are likely to impact cattle industries in adjacent states and beyond; it is my sincere hope that the data presented in this dissertation are of use to producers, veterinarians, and diagnosticians in those areas, as well.

The following chapters are published manuscripts detailing the initial description of *T. orientalis* Ikeda in the United States, and a second manuscript describing identification of co-infections of *T. orientalis* complex and *A. marginale* in the state of Virginia.

Hypothesis and Specific Aims

Hypothesis: *Theileria orientalis* Ikeda genotype/genotype 2 and Chitose genotype/genotype 1 each co-infect Virginia cattle with *Anaplasma* marginale.

Specific aim 1: Identify that *T. orientalis* Ikeda genotype/genotype 2 is in cattle in Virginia.

Specific aim 2: Determine whether co-infection of *T. orientalis* Ikeda and *Anaplasma marginale* can occur.

Specific aim 3: Determine whether co-infection *T. orientalis* Chitose and *Anaplasma marginale* can occur.

Chapter 1: *Theileria orientalis* Ikeda Genotype in Cattle, Virginia, USA

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Abstract. *Theileria orientalis* Ikeda genotype is a parasite that causes a disease in cattle that results in major economic issues in Asia, New Zealand, and Australia. The parasite is transmitted by *Haemaphysalis longicornis* ticks, which have recently been reported in numerous states throughout the eastern United States. Concurrently, cattle in Virginia showed clinical signs consistent with a hemoprotozoan infection. We used amplicons specific for the major piroplasm surface protein and small subunit rDNA of piroplasms to test blood samples from the cattle by PCR. Bidirectional Sanger sequencing showed sequences with 100% identity with *T. orientalis* Ikeda genotype 2 sequences. We detected the parasite in 3 unrelated herds and from various animals sampled at 2 time points. Although other benign *T. orientalis* genotypes are endemic to the United States, detection of *T. orientalis* Ikeda genotype might represent a risk for the cattle industry in Virginia.

Keywords: Theileria orientalis, Ikeda genotype, parasites, theileriosis, cattle, infectious disease, ticks, Haemaphysalis longicornis, Ixodidae, anemia, zoonoses, tick-borne infections, vector-borne infections, Virginia, United States

Theileria orientalis is an emerging parasitic pathogen of cattle that was originally identified in the Eastern Hemisphere (1). The taxonomy of this group is evolving (2–4); taxonomic classification relies on sequencing of 2 major genes: the small ribosomal subunit (SSU) and the major piroplasm surface protein (MPSP). Use of the names of *T. orientalis* genotypes Ikeda, Chitose, and Buffeli is embedded in the clinical literature; these designations are used throughout this article. A genotype scheme proposed in 2014 classifies *T. orientalis* into 11 genotypes according to variability in the MPSP gene (4); *T. orientalis* genotype Ikeda correlates with genotype 2 (3,5).

Consistent with other members of the genus, *T. orientalis* is a tickborne hemoprotozoan with a life cycle that affects erythrocytes and leukocytes and contributes to chronic anemia, ill-thrift, and persistent subclinical infections. However, it has not been associated with the lymphoproliferative disease seen with *T. parva* and *T. annulata* (1). *Haemaphysalis* spp. ticks are the primary biological vector of *T. orientalis* (6) and are believed to be essential for completion of the *T. orientalis* life cycle (7), although there is limited evidence suggesting that transmission might occur through flies, lice, or vaccine needles (1).

In Asia, New Zealand, and Australia, theileriosis caused by *T. orientalis* is an economically serious disease manifested primarily by loss of revenue from deaths or illness in beef and dairy cattle (1,8–10). Of increasing concern is the Ikeda genotype of *T. orientalis*, which has been implicated as the etiologic agent of infectious bovine anemia (11,12). In Asia, Australia, and New Zealand, the primary tick vector for the *T. orientalis* Ikeda genotype is *Haemaphysalis longicornis*, which is also known as the Asian longhorned or bush tick.

The Asian longhorned tick was first detected in the United States in August 2017 and has subsequently been detected in New Jersey (13), New York, North Carolina, Virginia, West Virginia, Pennsylvania, Maryland, Connecticut, and Arkansas (14,15). However, examination of archived tick samples has identified *H. longicornis* ticks in the United States since 2010 (14). Because of the wide host range of this tick, its

bisexual nature, and its ability to reproduce parthenogenetically (*16*), concern is increasing that there are established populations in the mid-Atlantic states.

In September 2017, a beef cattle herd in Virginia was given a diagnosis of anemia and suspected anaplasmosis. Blood samples were negative for *Anaplasma marginale* ticks by PCR, but blood smears showed numerous pleomorphic piroplasms. We report identification and characterization of *T. orientalis* Ikeda genotype 2 from an index farm, in adjacent herds, and 2 other counties in Virginia. Although other genotypes of *T. orientalis* are present in the United States (*17*–*19*), the Ikeda genotype in particular has not been identified in North America and represents an emerging infectious disease with potential for major animal health and economic impacts, especially because a competent vector has been identified in this region and in 2 of the 3 farms described in this report.

Material and Methods

Animals

All cattle were client-owned animals. In August 2017, seven cattle from a herd in Albemarle County, Virginia, died after showing adverse clinical signs, including weakness and malaise. Affected cattle included bulls, cows, and steers ranging in age from 3 months to 13 years. All animals were born and raised on a farm. In September 2017, an additional cow from the index farm was examined for weakness, icterus, and anemia (packed cell volume [PCV] 12.0%). Blood from this animal was collected and submitted to the Kansas State Veterinary Diagnostic Laboratory (Manhattan, KS, USA). Blood smear analysis showed evidence of a hemoprotozoal infection (Figure 1). Molecular testing of this sample resulted in a diagnosis of infection with *T. orientalis*, which prompted quarantine of the affected farm and further investigation.
A foreign animal disease investigation was instituted during December 2017, and blood was collected from the index cow and 5 additional, randomly sampled cattle from the herd. We collected blood by jugular vein venipuncture from each animal in 10-mL BD Vacutainer plastic red-top tubes containing no anticoagulant and in 10-mL BD Vacutainer plastic purple-top tubes containing EDTA anticoagulant (both from Becton,

Dickinson and Company, https://www.bd.com) as part of a routine diagnostic investigation for suspected anaplasmosis or as part of a random sampling effort.

DNA Extraction and PCR Testing

We extracted DNA from EDTA anticoagulant blood by using the DNeasy Blood and Tissue Kit (QIAGEN, https://www.qiagen.com) according to the nonnucleated blood protocol. We performed the final elution stage by using 50 µL of nuclease-free water and incubating the spin column membrane for 1 min at room temperature. This step was repeated to yield a final elution volume of 100 µL.

We performed amplification for the MPSP by using 10.5 µL of DNA in a 25-µL reaction volume containing primers MPSP forward (5′-CTTTGCCTAGGATACTTCCT-3′) and MPSP reverse (5′-ACGGCAAGTGGTGAGAACT-3′) as described (*11*,*20*). Each amplification had a final reaction primer concentration of 0.4 µmol/L.

We performed amplification for the internal segment of the SSU by using 10 µL of DNA in a 25-µL reaction volume containing primers SSU internal forward (5′-ATTGGAGGGCAAGTCTGGTG-3′) and SSU internal reverse (5′-CTCTCGGCCAAGGATAAACTCG-3′) as described (*11*,*20*) and identical PCR protocols as we described previously. We also used a Biometra TProfessional Thermocycler (AnalytikJena AG, https://www.analytik-jena.com).

We visualized amplicons by electrophoresis on a 1.0% agarose gel containing ethidium bromide and Tris-borate-EDTA buffer and examined amplicons by using UV transillumination. Amplicons with sizes of 700–800 bp were submitted to the Virginia Biocomplexity Institute (Blacksburg, VA, USA) for bidirectional Sanger sequencing.

Anaplasmosis Testing

Animals sampled as part of a foreign animal disease investigation were tested for *Anaplasma*, *Babesia*, and *Leptospira* spp. at the National Veterinary Services Laboratories (NVSL; Ames, IA, USA). The other animals involved in this study were tested for *Anaplasma marginale* by using a

quantitative PCR used and validated by the Virginia Tech Animal Laboratory Services (ViTALS, Blacksburg, VA, USA) diagnostic laboratory (*21*) (Table).

For the samples tested by ViTALS, we extracted DNA from blood by using the protocol we described previously. DNA samples were diluted 1:10 with nuclease-free water. Amplifications were performed by using AM-For 16S forward primer (5′-TTGGCAAGGCAGCAGCTT-3′) and AM-Rev 16S reverse primer (5′-TTCCGCGAGCATGTGCAT-3′) at a concentration of 0.6 μmol/L each, and AM-Pb probe (5′-6-FAM/TCGGTCTAACATCTCCAGGCTTTCAT/3BHQ_1-3′) at a concentration of 0.2 μmol/L. Reactions were performed in an ABI 7500 Fast thermocycler (Thermo Fisher Scientific, https://www.thermofisher.com) as described (*21*).

Sequence Analysis

We examined Sanger sequences of SSU and MPSP amplicons for quality and integrity and generated a consensus sequence by using Geneious Prime R11 (Biomatters, https://www.geneious.com). For paired samples of insufficient quality to form a consensus sequence, the highest quality sequence of the 2 sequences was used. We then aligned consensus sequence extractions with sequences of 3 *T. orientalis* Ikeda genotypes (GenBank accession nos. AB581627, AP011946, and D11046) by using Geneious Prime.

Phylogenetic Analysis

To examine the phylogenetic relationship of the cattle parasite in Virginia with other *Theileria* species, including the 3 genotypes of *T. orientalis*, we constructed a neighbor-joining tree by using a Tamura–Nei genetic distance model with 100 replications. We used the phylogenetic tree for the MPSP gene to best illustrate the relatedness of the samples from cattle in Virginia to the described *T. orientalis* genotypes (*4*). Phylogenetic analysis for the SSU gene included the *T. orientalis* Chitose genotype (GenBank accession no. AB520954), 2 *T. orientalis* Buffeli genotypes (accession nos. AB520955–6), 2 *T. orientalis* Ikeda genotypes (accession nos. AB520957–8), and *T. annulata* (accession no. AY524666) and *T. parva* (accession no. AF013418) as outgroups.

Results

Animals

Serologic analysis performed at NVSL for the 6 cattle tested showed negative results for *Anaplasma*, *Babesia*, and *Leptospira* species; all 6 animals were infected with piroplasmid hemoparasites on the basis of blood smear review. Subsequent blood samples were collected during May 2018 from 7 animals within the index herd. Two of these animals previously had intracellular piroplasms; 5 of these animals were sampled randomly. Multiple cattle were infested with ticks, which were morphologically identified as *H. longicornis* by NVSL (*14*). In July 2018, blood samples were collected from 21 cattle representing animals from the index herd and others owned by the same producer on other farms in the county. Before the day of collection, there had been no contact among cattle from different herds. The 21 samples collected represented blood from 20 previously unsampled animals (sampled randomly) and 1 from a calf that had been piroplasm positive during May 2018.

A random sample from a livestock auction from a cow in Albemarle County was submitted during August 2018 (Al1). Four additional samples were submitted from animals in Augusta (A1, A2), Pulaski (P1), and Albemarle (Al2) Counties during October 2018. These animals were unrelated to the cattle at the index farm and had no contact with each other. Of these 5 samples, 3 samples were random samples from a livestock auction (A1, A2, Al1), and 2 samples were submitted for evaluation of clinical disease (P1, Al2). All animals, except P1, were negative for *Anaplasma marginale*. On the basis of a blood smear, P1 had 16% of its erythrocytes parasitized by piroplasms (Table). In addition, this animal had a marked macrocytic, hypochromic, regenerative anemia, hyperbilirubinemia, and icteric plasma consistent with hemolytic anemia (PCV 14.4% [reference range 24.0%–46.0%]; mean corpuscular volume 94.4 fL [reference range 40.0 fL–60.0 fL]; mean corpuscular hemoglobin concentration 27.3 g/dL [reference range 30.0 g/dL–36.0 g/dL]; reticulocytes 194,400/µL [reference range not established]; and total bilirubin 4.1 mg/dL [reference range 0.1 mg/dL–0.6 mg/dL]). Blood smear analysis revealed a 16% parasitemia. P1 died shortly after the blood

draw; another animal on the farm had icterus and died 3 days earlier. Al2 had a PCV of 10.0% and showed signs of icterus and lethargy.

Blood Smears

The blood smear examinations for the cow that became ill during September 2017 showed evidence of a regenerative response to anemia (anisocytosis, polychromasia, basophilic stippling) and numerous oval, racquet, signet ring, and linear piroplasms within erythrocytes (Figure 1). During May 2018, blood smear analysis on 7 additional, randomly sampled animals did not show piroplasms. Two of these samples were from animals that had blood collected and analyzed by NVSL during December 2017; both of these animals were positive for piroplasms in blood smears at that time.

PCR

Molecular testing of the initial blood sample from the cow that became ill during September 2017 (I1) resulted in a diagnosis of infection with *T. orientalis*. This finding, which, in conjunction with the severity of the clinical signs in this animal and death of previous animals on the premises, led to further investigation of the genotype of the organism and quarantine of the affected farm.

Five of the 6 blood samples taken from cattle during December 2017 were positive by MPSP or SSU assays. Six of 7 cattle from the index herd sampled during May 2018 were positive by MPSP and SSU assays. Of the additional 21 samples collected during July 2018 from the index herd and adjacent properties, only samples from 3 animals were negative by MPSP and SSU assays. Three cattle from Augusta and Pulaski Counties (A1, A2, P1) and 2 cattle from an unrelated herd in Albemarle County (Al1, Al2) were positive by MPSP and SSU assays. A total of 34 sequences were available for analysis, representing 31 unique animals from multiple farms across southwestern Virginia.

Two cattle (I2 and I3) were positive by MPSP and SSU assays during December 2017, and remained positive by these assays during May 2018. One animal was positive by MPSP and SSU assays during May

2018, and remained positive for both assays during July 2018. The presence of positive samples at a sampling interval of 1–5 months suggests a chronic or persistent component of the infection.

Sequence and Phylogenetic Analysis

All 34 MPSP gene (719 bp) sequences from cattle from multiple farms in all 3 counties in Virginia sampled were identical to each other and to *T. orientalis* Ikeda and genotype 2 sequences from GenBank (Figure 2). MPSP sequences of animals I2 and I3 sampled during December 2017 and May 2018 were identical.

On the basis of MPSP gene sequence phylogeny reported by Sivakumar et al. (*4*), all clinical samples clustered together, along with *T. orientalis* Ikeda and genotype 2 sequences retrieved from GenBank (Figure 3). The next closest related branch was composed of the cluster of *T. orientalis* genotype 7. Outgroups composed of *T. annulata* and *T. parva* clustered appropriately. On the basis of SSU gene sequence phylogeny (*4*,*22*) all clinical samples clustered together, along with *T. orientalis* Ikeda genotype sequences from GenBank (Figure 4).

Discussion

Genotypes of *Theileria orientalis* are native to the United States, but are of the Buffeli genotype and are typically nonpathogenic (*17*–*19*). *T. orientalis* Ikeda/genotype 2, a novel, virulent genotype, has not been previously identified in North America. Although the vector of *T. orientalis* among cattle in Virginia is unknown, *H. longicornis* ticks are a major vector in New Zealand, Australia, and Asia (*1*). The recent identification of this tick in the United States and clinical signs of anemia in an *Anaplasma*-negative herd of cattle infested by *H. longicornis* ticks prompted further investigation into the genotype of *T. orientalis* detected in the cattle sampled.

MPSP is an antigenic marker of *Theileria* spp., and is used to genotype the 11 *Theileria* groups (*4*,*5*). The sequences analyzed in this study are phylogenetically consistent with genotype 2, equivalent to the Ikeda genotype. Because the samples examined in this study represent individual cattle from geographically distant herds, there is concern that *T. orientalis* Ikeda/genotype 2 could be widespread in the region. This

concern is especially potent given the presence of a known vector, *H. longicornis* ticks, within the region simultaneously (*14*). No ticks from the region have been tested for *T. orientalis*. Thus, further work is needed to better understand transmission of *T. orientalis* Ikeda by *H. longicornis* ticks or other tick species in Virginia.

The presence of *T. orientalis* Ikeda/genotype 2 in Augusta County is of particular concern because this county has the second largest number of cattle in Virginia (*23*). Rockingham County, which produces most of the cattle in Virginia, is adjacent to Augusta and Albemarle Counties. Future studies are needed to determine the presence of *H. longicornis* ticks and *T. orientalis* Ikeda/genotype 2 in this area, but disease transmission to and within this area is of concern to cattle producers in Virginia. In countries in which *T. orientalis* Ikeda is established, the parasite contributes to economic losses through chronically ill animals. During 2010 in Australia, losses caused by *T. orientalis* Ikeda were estimated to be Aus ≈$20 million (*5*,*10*). In New Zealand, the cost of 1 outbreak on a large dairy farm was estimated to be ≈1 million New Zealand dollars (*5*).

In this initial study of cattle in the United States, some of the animals that were positive for *T. orientalis* Ikeda by PCR remained positive 5 months later, suggesting a chronic state, although the animals that had initially exhibited clinical signs were no longer clinically ill during the spring and summer months. In New Zealand, disease caused by *T. orientalis* demonstrates seasonal pathogenicity, and anemia is more pronounced during autumn and winter months (*12*). Whether this trend is the case in Virginia will require future exploration. These cattle were also initially suspected to have anaplasmosis and had additional testing not been conducted, the detection of this pathogenic *Theileria* species might not have occurred. This study highlights the need for more surveillance and appropriate characterization of any parasites detected.

The source and timing of introduction of *T. orientalis* Ikeda/genotype 2 into the United States is unclear. One potential mechanism of introduction is through subclinically infected live cattle imported from disease-endemic regions. Approximately 200 live Wagyu cattle were imported from Japan during 1993–1997 before

a ban on exports of Wagyu cattle by Japan in 1997 (*24*). Some of these same cattle were exported to Australia. It is possible that this or other similar live cattle trade introduced this genotype to the United States. Another possibility is transstadial transmission of *H. longicornis* ticks accidentally brought into the United States on other animals in shipments. Other genotypes of *T. orientalis* have been identified in the United States (*14*,*17*,*18*), suggesting that there were previous introductions. Regardless, now that both the tick and the hemoprotozoan are established in this region, *H. longicornis* ticks will probably play a role in continued transmission of the parasite, and *Theileria*-associated bovine infectious anemia will likely continue to occur within the area. A study modeling the most likely suitable habitats of the Asian longhorned tick throughout North America predicts that the tick will thrive along the eastern United States seaboard from Maine to North Carolina, along the western United States seaboard from Washington to northern California, and from northern Louisiana northward into Wisconsin and Ohio (*25*). With such a broad range, we predict *T. orientalis* Ikeda will become established in multiple states throughout the country.

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Figure 1 Blood smear of an animal from a farm in Albemarle County, Virginia, USA, that was infected with *Theileria orientalis* Ikeda genotype. There is evidence of a regenerative response to anemia (anisocytosis and polychromasia) and intracellular piroplasms within erythrocytes. Scale bar indicates 10 µm.

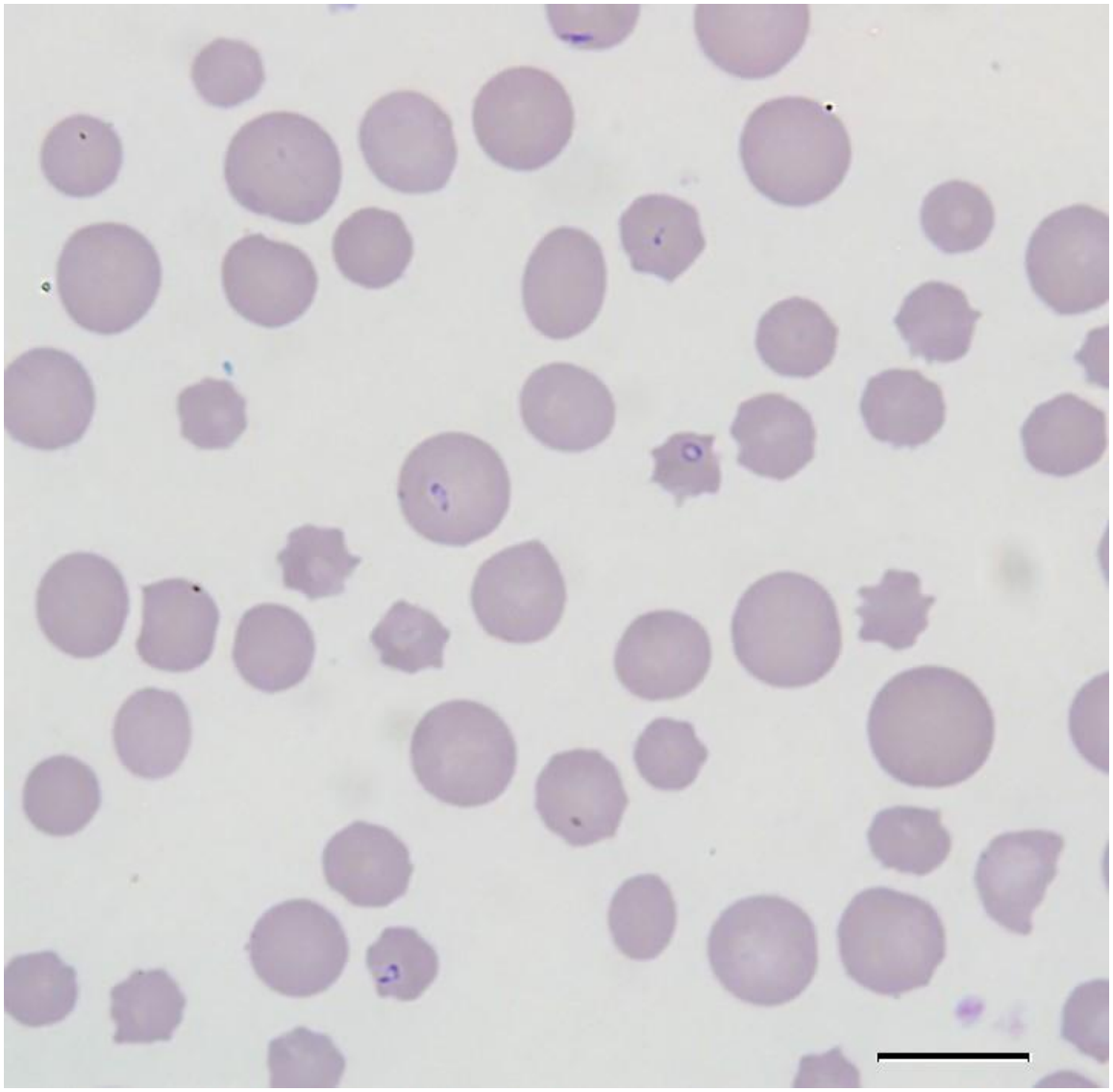

Figure 2. DNA sequences from 10 cattle from a farm in Albemarle County, Virginia, USA, infected with *Theileria orientalis* Ikeda genotype aligned with 3 GenBank sequences of *T. orientalis* genotype 2 for the major piroplasm surface protein. Alignment shows 100% consensus. Samples represent cattle from 6 different herds, and 2 samples were obtained at 2 time points. Pink indicates adenine, yellow indicates guanine, green indicates thymine, and purple indicates cytosine. Nucleotides at the top indicate the consensus sequence. The GenBank sequence THEPMiPI is RNA with uracil substituted for thymine.

Figure 3. Phylogenetic tree showing major piroplasm surface unit gene sequences for *Theileria* species. The tree uses reference sequences from the major genotypes for *T. orientalis* (4). Sequences from infected cattle in Virginia, USA, cluster with genotype 2 sequences. Numbers along branches are bootstrap values. Scale bar indicates nucleotide substitutions per site.

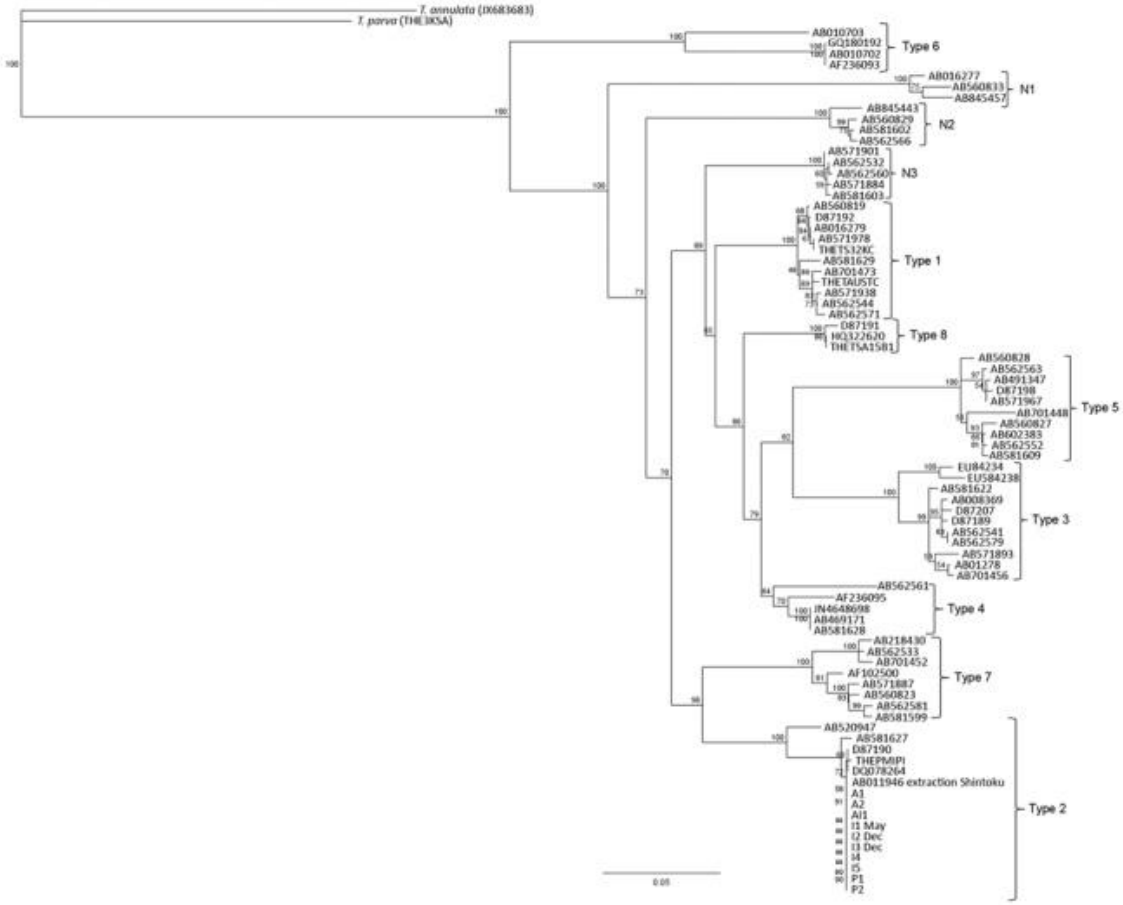

Figure 4. Phylogenetic tree showing small subunit rDNA gene sequences for *Theileria* species. Representative samples from cattle in 6 herds in Virginia, USA, cluster with the reference sequences for the Ikeda genotype. The next most closely related branch is composed of *T. orientalis* Chitose genotype and then *T. orientalis* Buffeli genotype. The outgroup is composed of a single reference sequence each for *T. parva* and *T. annulata*. Small subunit rDNA sequences for I4 and Al1 were of low quality (6.4% and 3.9%, respectively). Numbers along branches are bootstrap values. Scale bar indicates nucleotide substitutions per site.

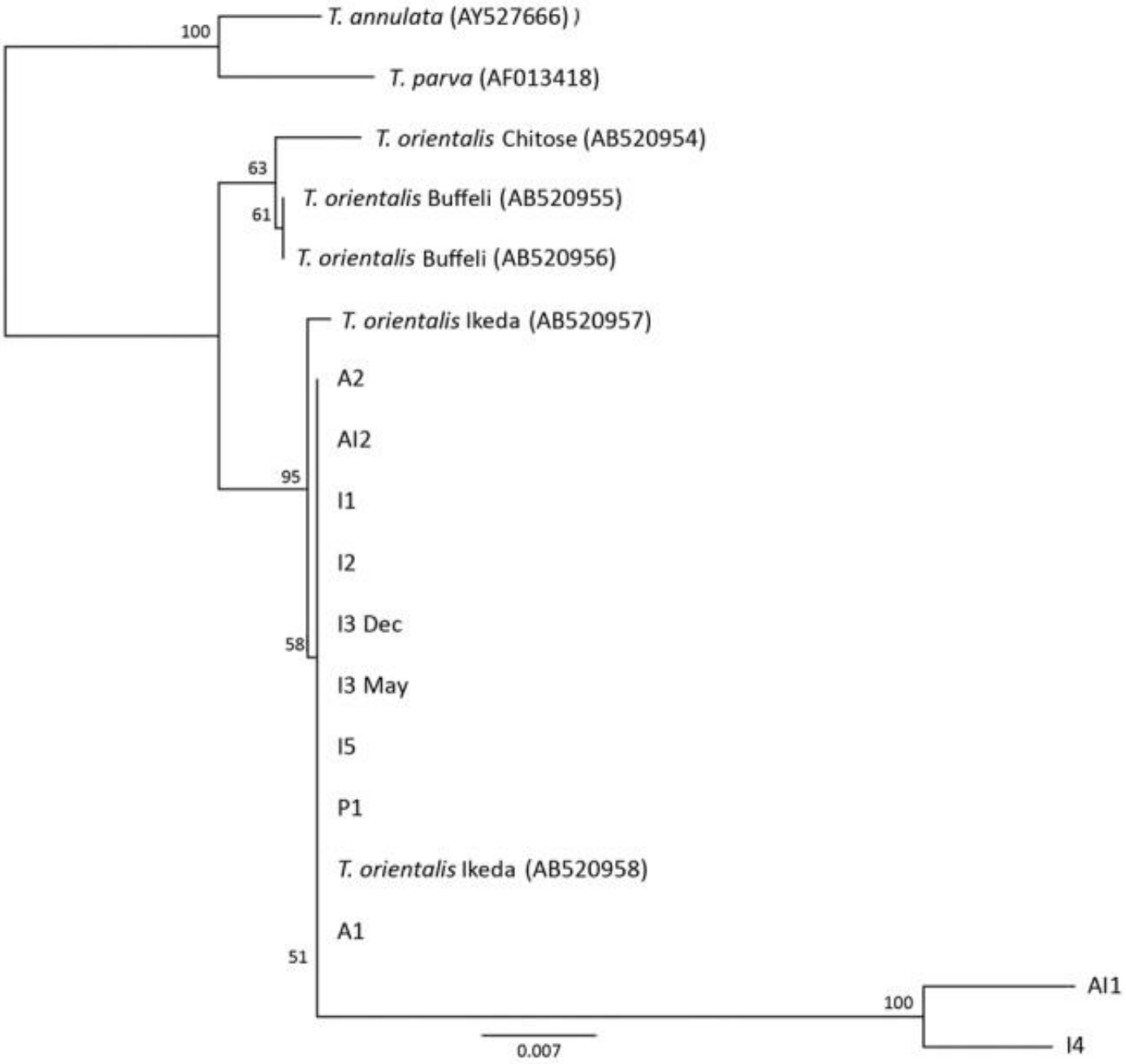
T. annulata (AY527666)
T. parva (AF013418)
T. orientalis Chitose (AB520954)
T. orientalis Buffeli (AB520955)
T. orientalis Buffeli (AB520956)
T. orientalis Ikeda (AB520957)
A2
AI2
I1
I2
I3 Dec
I3 May
I5
P1
T. orientalis Ikeda (AB520958)
A1
AI1
I4
100
63
61
95
58
51
100
0.007

Table 1. Diagnostic testing results for *Theileria orientalis* Ikeda genotype in cattle, Virginia, USA*

Animal ID	Icterus/PCV*/Parasitemia	Anaplasma	Origin	Date
I1	Yes / 12.0% / 6%	Negative (NVSL*)	Albemarle (Index farm)	Sep. 2017 Dec. 2017
I2	No	Negative (NVSL)	Albemarle (Index farm)	Dec. 2017 May 2018
I3	No	Negative (NVSL)	Albemarle (Index farm)	Dec. 2017 May 2018
I4	No	Negative (ViTALS*)	Albemarle (Index farm)	July 2018
I5	No	Negative (ViTALS)	Albemarle (Index farm)	July 2018
Al1	No	Negative (ViTALS)	Albemarle	Aug. 2018
Al2	Yes / 10.0% / NA*	Negative (ViTALS)	Albemarle	Oct. 2018
P1	Yes / 14.4% / 16.4%	Positive (ViTALS)	Pulaski	Oct. 2018
A1	No	Negative (ViTALS)	Augusta	Oct. 2018
A2	No	Negative (ViTALS)	Augusta	Oct. 2018

* ID, identification; PCV, packed cell volume; NA, not available; NVSL, National Veterinary Services Laboratories; ViTALS, Virginia Tech Animal Laboratory Services.

Chapter 2: Coinfection of cattle in Virginia with *Theileria orientalis* Ikeda genotype and *Anaplasma marginale*

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Abstract. *Theileria orientalis* Ikeda is a newly identified agent of bovine infectious anemia in the United States. Although *T. orientalis* Ikeda is transmitted by ticks other than the tick that transmits *Anaplasma marginale*—a bacterial etiology of bovine infectious anemia—the geographic distributions of these 2 infectious organisms overlap, with coinfection reported in some cattle. Only anaplasmosis has an approved effective treatment in the United States. To provide rapid diagnostic information for producers with anemic animals, we developed a duplex real-time PCR (rtPCR) for *A. marginale* and *T. orientalis*. With a cutoff of 38 cycles, the duplex assay has a sensitivity of 97.0% and a specificity of 100% for *A. marginale*; with a cutoff of 45 cycles, the duplex assay has a sensitivity and a specificity of 100% for *T. orientalis*, compared to existing tests. In addition to providing a tool for improved clinical decision-making for veterinarians and producers, our rtPCR facilitates the study of coinfection of cattle in Virginia. Of 1,359 blood samples analyzed, 174 were positive for *T. orientalis*, 125 were positive for *A. marginale*, and 12 samples were positive for both *T. orientalis* and *A. marginale.* Hence, coinfection by these 2 agents of bovine infectious anemia does occur within Virginia. It is likely that this pattern of infection will be seen in other regions where *T. orientalis* and *A. marginale* infections are endemic, despite the difference in tick vectors.

Keywords: *Anaplasma marginale*; bovine infectious anemia; *Theileria orientalis* Ikeda.

The *Theileria orientalis* complex describes several genotypes of a species of non-transforming, theilerial hemoprotozoan. Within the complex, the Ikeda and Chitose genotypes (*T. orientalis* Ikeda and *T. orientalis* Chitose, respectively) are capable of causing disease. *T. orientalis* Ikeda in particular is increasingly implicated as a causative agent of bovine infectious anemia in the United States, a vector-borne disease characterized by hemolytic anemia, icterus, general malaise, ill thrift, and sporadic abortions.[9,11,12] Although rarely fatal, affected animals are often poorly producing; consequently, this is a disease of economic importance in the countries in which it is found.[9,11]

T. orientalis is transmitted most effectively by the Ixodidae tick *Haemaphysalis longicornis*,[10] a tick that has recently been discovered in several states along the U.S. Eastern Seaboard.[16] *T. orientalis* Ikeda has recently been identified in cattle in Virginia, affecting animals that were also parasitized with *H. longicornis* ticks[12]; this tick has been confirmed as a competent vector in Virginia for *T. orientalis* Ikeda.[8] The animals that were clinically affected in this outbreak had the typical signs of anemia, icterus, and general malaise. In Virginia, this clinical presentation is identical to the blood infection caused by the bacterium *Anaplasma marginale. H. longicornis* is not capable of transmitting *A. marginale*,[6] and in many other parts of the world, the geographic distribution of *T. orientalis* Ikeda and *A. marginale* do not overlap (Jenkins C, pers. comm., 2021 Jun 10). In Virginia, however, the initial outbreak of *T. orientalis* Ikeda occurred in areas where anaplasmosis has been diagnosed historically, suggesting an overlap in geographic distribution in this region. Further, the predicted range of *H. longicornis* based on modeling suggests its presence in areas of the country where anaplasmosis is common.[14] An animal included in the initial outbreak study in Virginia was positive for both *T. orientalis* Ikeda and *A. marginale* by conventional PCR and Sanger sequencing.[12] As a bacterial infection, anaplasmosis is treatable using oxytetracycline, a cost-effective drug approved for use in food animals. Theileriosis is markedly more difficult to treat, and no effective drugs are approved for use in food animals in the United States, although buparvaquone has been effective in Australia.[5] Therefore, making an early distinction between anaplasmosis and theileriosis is critical for immediate and effective

clinical decision-making that has animal welfare, productivity, and economic implications. To be of use to producers, it is important that the tools used to assist in this process are cost-effective.

In response, we developed a duplex real-time PCR (rtPCR) assay that detects both *A. marginale* and *T. orientalis*. Multiplex assays that can detect *Theileria* spp. and *Babesia* spp. protozoans, and *Anaplasma* spp. bacteria, have been developed for small ruminants[1,7]; we sought to fill the niche for cattle. We developed our assay in accordance with published standards.[15] Although analytical sensitivity was determined by examining the limit of detection (LOD), the reported specificity and sensitivity for *A. marginale* and *T. orientalis* are relative to the specificity and sensitivity of the tests against which they were compared. For *T. orientalis* in particular, there are few validated tests against which to compare, hence the interpretation of diagnostic sensitivity (DSe) and diagnostic specificity (DSp), although promising, must take that into consideration.

Materials and methods

Blood

Whole blood samples were submitted from privately owned cattle across the state of Virginia. Animals included clinical submissions by private veterinarians, as well as herds owned and maintained by the Virginia Department of Corrections (VADOC), and as part of an ongoing surveillance effort by the Virginia Department of Agriculture and Consumer Services (VDACS) of locally produced animals sent to auction. Whole blood was collected in K_2-EDTA blood collection tubes (Vacutainer; Becton, Dickinson). Except for animals submitted by referring veterinarians as part of a routine clinical diagnostic workup, all animals were randomly sampled at auction houses, and the presence and degree of clinical signs were unknown. Convenience-sampled animals were submitted as part of an ongoing surveillance effort by VDACS. These samples were taken from cattle at the time of auction. A number of animals from each pen within the auction house were selected for a blood draw, regardless of the presence or absence of clinical signs and attempting to avoid collecting samples from animals from the same herd. Although the samples themselves

were collected in limited geographic locations (the auction houses), they represented animals from herds across Virginia. In total, 1,359 blood samples were available for evaluation.

DNA extraction

DNA was extracted from K_2-EDTA anticoagulated blood (DNeasy blood and tissue kit; Qiagen) following the manufacturer's protocol with modifications.[11] The initial blood volume for DNA extraction was 100 µL, and control DNA (Applied Biosystems VetMAX Xeno internal positive control DNA; Thermo Fisher) was added to the lysis buffer of the Qiagen kit (Buffer AL) at 20,000 copies per sample. DNA was eluted twice in 50 µL of Buffer AE of the Qiagen kit, pre-heated to 56°C, for a total elution volume of 100 µL. All extractions were carried out within a biosafety cabinet.

Duplex rtPCR

Our duplex assay is a TaqMan-based assay that utilizes primers and probes designed to detect the major surface protein 1b (*msp1b*) gene of *A. marginale*, and the major piroplasm surface protein (*MPSP*) gene of *T. orientalis*. The primers and probes for *MPSP* are sensitive for *T. orientalis* but are not genotype-specific; to further characterize genotype, a second assay is run on those samples that are positive for *T. orientalis*.

The gene target for *A. marginale*, *msp1b*, is a highly expressed, conserved gene that persists between tick and cattle transmission cycles and is the target of an *A. marginale*–specific rtPCR.[3] The *MPSP* gene of *T. orientalis* was chosen because it is the major antigenic target and the gene used for genotyping the *T. orientalis* complex.

Amplification for the *msp1b* and *MPSP* genes were accomplished in the same reaction. The duplex rtPCR reaction consisted of 10 µL of master mix (Applied Biosystems TaqMan environmental master mix 2.0; Thermo Fisher), 0.6 µL of each *T. orientalis* forward and reverse primer, 1.2 µL of each *A. marginale* forward and reverse primer, 0.2 µL of *T. orientalis* universal probe, 0.4 µL of *A. marginale* probe, 0.8 µL of primer–probe mix (Xeno VIC; Thermo Fisher), and 2 µL of DNA template in a 20-µL reaction. The primers and probes for *T. orientalis* and *A. marginale* have been published previously.[2,4] Fluorophores and quenchers were altered from the published probes to allow appropriate multiplexing (Table 1). The probes

utilize 3 separate fluorescent tags: FAM for *A. marginale*, NED for *T. orientalis*, and VIC for the internal positive control. Amplification was completed (Applied Biosystems 7500 fast real-time PCR system; Thermo Fisher) using standard cycling and the following run method: 95°C for 10 min, followed by 45 cycles composed of 15 s of 95°C for annealing, and 1 min of 60°C for extension. Reactions were completed (Applied Biosystems MicroAmp Fast 8-tube strips, Applied Biosystems MicroAmp Optical 8-cap strips; Thermo Fisher). A sample lacking DNA template was included as a negative control with each run. A *T. orientalis* known-positive sample, an *A. marginale* known-positive sample, and 2,000 copies of internal positive control DNA (VetMAX Xeno; Thermo Fisher) were included in separate reactions with each run to serve as positive controls for the respective fluorophore channels and negative extraction controls for the other targets.

Validation: limit of detection

To determine the LOD for analytic sensitivity for *T. orientalis*, 3 identical rtPCR runs were completed per the protocol described above. Eight, 10-fold serial dilutions of gBlock gene fragments (Integrated DNA Technologies) with the universal *MPSP* gene sequence capable of detecting each of the genotypes Ikeda, Chitose, and Buffeli were used. The LOD was determined as the lowest dilution at which samples on all 3 plates were positive, and a LOD was determined for each of the 3 *T. orientalis* genotypes of interest. Similarly, the LOD was determined for *A. marginale* using serial dilutions of an *A. marginale*–positive blood sample confirmed positive by external rtPCR and shown to have 1.22% infected RBCs.

Validation: repeatability

For our intra-assay repeatability test of precision, we examined the high (undiluted), medium (1:100 dilution), and low (1:10,000 dilution) concentrations of reference DNA in quintuplicate in one run on one day. Reference DNA was extracted from samples confirmed positive on in-house PCR. In this assay, the average cycle threshold (Ct) was compared to the SD of the 5 replicates of a given concentration.

For our inter-assay repeatability tests, we examined high, medium, and low concentrations of reference DNA in quintuplicate on each of 6 consecutive days, in accordance with OIE recommendations.

Inter-assay repeatability was calculated by plotting the Ct value versus the day. We used one-way ANOVAs of both inter- and intra-assay repeatability tests to examine the difference in Ct means across days or within a single run, respectively. All repetitions were performed on the same thermocycler used above.

Relative diagnostic sensitivity and specificity

The relative DSe and DSp of the *T. orientalis* and *A. marginale* targets within the duplex assay were determined with receiver operating characteristic (ROC) statistics. Our genotype-specific *T. orientalis* rtPCR described below was used as the reference standard test against which the duplex rtPCR results were compared. For *A. marginale*, rtPCR was completed either at the U.S. Department of Agriculture's Agricultural Research Service, Animal Disease Research Unit (USDA-ARS-ADRU; Pullman, WA, USA) or at the Washington Animal Disease Diagnostic Laboratory (WADDL; Washington State University, Pullman, WA, USA) as the reference standard test.

Genotyping rtPCR

A second multiplex assay that utilizes distinct probes capable of differentiating the *MPSP* gene of 3 of the major *T. orientalis* genotypes—Chitose, Ikeda, and Buffeli—further characterized the genotype. The forward and reverse primers are the same as those used for *T. orientalis* in the duplex rtPCR protocol above, but with the use of a distinct set of probes to distinguish among the genotypes[2] (Table 2).

DNA extracted from whole blood samples that were positive for *T. orientalis* on the duplex rtPCR were used for the genotype multiplex rtPCR. The 20-µL reactions consisted of 10 µL of master mix (Applied Biosystems TaqMan environmental master mix 2.0; Thermo Fisher), 0.6 µL of each *T. orientalis* forward and reverse primer, 0.5 µL of Ikeda probe, 0.2 µL of Chitose A probe, 0.3 µL of Chitose B probe, 0.2 µL of Buffeli probe, and 2 µL of DNA (Table 2). The instrumentation, run method, and plastics were the same as for the duplex rtPCR assay. A sample lacking DNA template was included with each run as a negative control. Positive controls for each of the 3 genotypes were included in each run, and runs were accepted only if controls worked as expected.

Genotyping

DNA that tested positive for the Chitose genotype of *T. orientalis* in the duplex rtPCR were subjected to 2 rounds of conventional PCR (cPCR) targeting the *MPSP* gene of *T. orientalis*. The reaction for the first round of cPCR consisted of DNA polymerase (Invitrogen platinum PCR supermix high fidelity; Thermo Fisher), 1 µL of each sense and anti-sense primer, and 10.5 µL of DNA in a 25-µL reaction volume. Primers (Integrated DNA Technologies) for *MPSP* were 5′-CTTTGCCTAGGATACTTCCT-3′ (sense) and 5′-ACGGCAAGTGGTGAGAACT-3′ (anti-sense).[11] The PCR program was as follows: 95°C for 2 min, following by 29 cycles consisting of 95°C for 15 s, 57°C for 30 s, 72°C for 1 min; after these 29 cycles, the program progressed to 72°C for 7 min, and then held at 12°C. A second round of cPCR was carried out using the amplicons from the first round as template, using the same reaction components and PCR program. Second round amplicons were electrophoresed on 1% agarose in Tris-borate with added ethidium bromide gel and visualized under UV light. DNA bands were purified from the gel (QIAquick gel extraction kit; Qiagen) and submitted to the Fralin Life Sciences Institute, Genomic Sequencing Center (Blacksburg, VA, USA), for Sanger sequencing. Sequences were then aligned to GenBank accessioned sequences for each of the Ikeda, Chitose, and Buffeli genotypes.

Results

Duplex rtPCR

Of the 1,359 individual DNA samples available for rtPCR testing, 186 (13.7%) were positive for *T. orientalis*, and 137 (10.1%) were positive for *A. marginale*. Included in these numbers are 12 samples (0.88%) that were positive for both *T. orientalis* and *A. marginale*.

Limit of detection

The LODs for *T. orientalis* genotypes Ikeda and Buffeli were 1×10^8 pmol of DNA. For the Chitose genotype, the LOD was 1×10^9 pmol. *A. marginale* was detected according to dilution rate, given the nature of the known-positive sample. The LOD of *A. marginale* was a 10^{-5} dilution of reference DNA, extracted from a clinically positive sample according to our in-house PCR. Although a standard curve was not run alongside this PCR assay, extrapolating from a similar assay run in our laboratory (supplementary data[8])

suggests that 1×10^9 pmol of Chitose gBlock is ~16 gene copies; 10^8 pmol of Ikeda gBlock is ~25 gene copies.

Repeatability

As calculated, there was significant variability between intra- and inter-assay repetitions of the *A. marginale* component of the assay. However, the variability did not impact the ultimate interpretation of the test results; negative results remained negative, and positive results remained positive (Table 3).

Relative diagnostic sensitivity and specificity

For *A. marginale*, the assay DSe was 97.0% and the DSp was 100% when a cutoff of 38 cycles was applied (Tables 4, 5). For *T. orientalis*, both the DSe and DSp were 100% when a cutoff of 45 cycles was applied (the final cycle of the rtPCR program; Tables 6, 7). The reference standard utilized for *A. marginale* was an external rtPCR, run either at the USDA-ARS-ADRU or WADDL.

The reference standard utilized for *T. orientalis* was the *T. orientalis* genotype rtPCR developed by the Virginia Tech Animal Laboratory Services (Virginia-Maryland College of Veterinary Medicine, Blacksburg, VA, USA), modified from a previous validated assay,[2] and described below.

Genotyping

Because a universal probe was used for the *MPSP* gene of *T. orientalis*, an additional genotyping assay was required for those samples that tested positive for *T. orientalis* in the duplex assay. The genotype multiplex assay was run using extracted DNA, applying the same *T. orientalis* forward and reverse primers, but utilizing genotype-specific *MPSP* probes. Samples of known genotype were included as positive controls. Of 186 *T. orientalis*–positive samples from the duplex PCR, 159 (85.5%) were consistent with the Ikeda genotype, 21 were consistent with the Chitose genotype, and the remaining 6 (3.2%) were undetermined. None were consistent with Buffeli. There were no mixed infections with multiple genotypes.

Discussion

The high DSe for *A. marginale* and *T. orientalis* makes our duplex rtPCR assay a useful screening tool for producers in Virginia and in other localities with concurrent occurrence of anaplasmosis and theileriosis.

There is reported evidence within the literature that the Chitose genotype is capable of causing disease on its own[10,11]; given that numerous cattle in our study sample were positive for Chitose alone, further investigation is warranted, not just into the geographic distribution and possible overlap of *A. marginale* and *T. orientalis* Ikeda, but also into the pathogenicity of the Chitose genotype. This is of particular importance for Virginia because *A. marginale* and pathogenic genotypes of *T. orientalis*—individually and in combination—have been found in counties that account for much of the state's cattle production. *T. orientalis* is a disease of economic importance in other countries in which it occurs, and being able to rapidly identify *A. marginale*, *T. orientalis*, or both agents in combination as the likely cause for clinical disease in cattle is important for determining treatment plans for veterinarians and producers.

A limitation of our experiment is the small number of reference tests available for *T. orientalis.* Because DSe and DSp of this proposed duplex PCR are calculated in reference to existing tests, those results rely heavily on the reference tests performing with high specificity and sensitivity. The reference test used for *A. marginale* was validated analytically at an AAVLD-accredited laboratory and published.[4] The reference test for *T. orientalis* was published,[12] but in addition, we confirmed a subset of the samples with cPCR and Sanger sequencing, improving confidence in the results. Our tool was designed to identify and distinguish between etiologic agents capable of producing identical clinical signs in cattle in Virginia, and, in this niche, it is fit-for-purpose.

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Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Table 1. Primer and probe sequences used for *Theileria orientalis* and *Anaplasma marginale* duplex PCR assay.

Target	Sequence
T. orientalis	
Forward primer	5′-GCAAACAAGGATTTGCACGC-3′
Reverse primer	5′-TGTGAGACTCAATGCGCCTAGA-3′
Universal probe	5′-NED-TCGACAAGTTCTCACCAC-MGB-NFQ-3′
A. marginale	
Forward primer	5′-TTGGCAAGGCAGCAGCTT-3′
Reverse primer	5′-TTCCGCGAGCATGTGCAT-3′

Table 2. Genotype-specific probe sequences used for the *Theileria orientalis* genotyping assay.

Probe target	Sequence
T. orientalis Ikeda	5′-VIC-CATGGACAGTGCTTGGC-MGB-NFQ-3′
T. orientalis Chitose A	5′-NED-TCCTCAGCGCTGTTCT-MGB-NFQ-3′
T. orientalis Chitose B	5′-NED-TCCTCGGCGCTGTTCT-MGB-NFQ-3′
T. orientalis Buffeli	5′-FAM-CTCCTTTGCAGTATTCTTCTATCTC-QSY-3′

Table 3. Comparison of one-way ANOVA results following the repeatability assay. The inter-assay repeatability was calculated by plotting the cycle threshold (Ct) value versus the day; intra-assay repeatability was calculated by plotting the Ct value versus the SD of 5 replicates of a given concentration.

Target/Concentration	One-way ANOVA	Variance
Anaplasma marginale		
Undiluted	<0.001	Yes
10^{-2}	0.003	Yes
10^{-4}	0.001	Yes
Theileria orientalis Buffeli		
Undiluted	0.783	No
10^{-1}	0.973	No
10^{-3}	0.548	No
T. orientalis Chitose		
Undiluted	0.278	No
10^{-1}	0.404	No
10^{-2}	0.409	No
T. orientalis Ikeda		
Undiluted	0.158	No
10^{-1}	0.737	No
10^{-2}	0.150	No

Table 4. A comparison of the diagnostic sensitivity (DSe) and diagnostic specificity (DSp) values for *Anaplasma marginale* compared to an external real-time PCR run at either USDA-ARS-ADRU or WADDL.

Cutpoint (Ct)	DSe	DSp
45.0	1.000	0.682
40.0	0.970	0.796
39.0	0.970	0.973
38.0	0.970	1.000
37.0	0.970	1.000
36.0	0.939	1.000

Ct = cycle threshold; USDA-ARS-ADRU = U.S. Department of Agriculture, Agricultural Research Service, Animal Disease Research Unit, Pullman, WA, USA; WADDL = Washington Animal Disease Diagnostic Laboratory, Washington State University, Pullman, WA, USA.

Table 5. Overview of the positive and negative cases for *Anaplasma marginale* compared to an external real-time PCR run at either USDA-ARS-ADRU or WADDL.

Cutpoint 38.00	
True positive	32
False positive	0
False negative	1
True negative	44
Total	77

USDA-ARS-ADRU = U.S. Department of Agriculture, Agricultural Research Service, Animal Disease Research Unit, Pullman, WA, USA; WADDL = Washington Animal Disease Diagnostic Laboratory, Washington State University, Pullman, WA, USA.

Table 6. A comparison of the diagnostic sensitivity (DSe) and diagnostic specificity (DSp) values for *Theileria orientalis* compared to a validated, in-house real-time PCR reference standard.

Cutpoint (Ct)	DSe	DSp
45.0	1.000	1.000
40.0	1.000	1.000
39.0	1.000	1.000
39.0	0.970	1.000
37.0	0.939	1.000
36.0	0.9	1.000

Ct = cycle threshold.

Table 7. Overview of the positive and negative cases for *Theileria orientalis* compared to a validated, in-house real-time PCR reference standard.

Cutpoint 45.00	
True positive	49
False positive	0
False negative	0
True negative	34
Total	83

Chapter 3: Co-infection of *Theileria orientalis* and *Anaplasma marginale* by genotype

Introduction

Following development of the *T. orientalis* and *A. marginale* duplex assay in Chapter 2, we were interested in determining whether co-infection with *T. orientalis* and *A. marginale* was limited to one genotype of *T. orientalis*. This hypothesis was partially inspired by preliminary data suggesting that *T. orientalis* Chitose appears to have a different geographic distribution than does Ikeda within Virginia (unpublished data). Although all current data out of the United States and in other countries indicates *H. longicornis* as the tick vector for Chitose, if the geographic distribution of *A. marginale*, Ikeda, and Chitose differ, it is possible there is an additional tick vector for Chitose not yet described in the US. Alternatively, perhaps environmental or topographical factors contribute to differential survival of *T. orientalis* genotypes.

Materials and Methods

Animals

We used the same database of DNA samples used for the *T. orientalis* and *A. marginale* duplex assay described in Chapter 2. Blood samples represent convenience sampled animals as part of ongoing VDACS surveillance efforts, convenience sampled animals from VDOC herds, or samples submitted as part of diagnostic investigations from producers and veterinarians throughout the state. All blood was submitted in EDTA anticoagulant. DNA was extracted using a modified protocol and a QIAGEN DNEasy Blood and Tissue Kit (QIAGEN, www.qiagen.com; Oakes et al. 2019). Except for those samples submitted specifically as part of a diagnostic investigation, all blood was convenience sampled, and the clinical status of animals unknown. Four-thousand, six-hundred and fifty-five samples were included in the study.

rtPCR Assay

The duplex assay developed in Chapter 2 was used. The target gene for *A. marginale* is the major surface protein 1b gene (msp1b) using the FAM fluorophore channel. The target gene for *T. orientalis* is the major piroplasm surface protein gene (mpsp) using the NED fluorophore channel. A VetMAX™ Xeno™ internal positive control was included, using the VIC fluorophore channel. All samples were run in an Applied

Biosystems™ 7500 Fast Real-time PCR system using the following runtime method: 95ºC for 10 minutes, followed by 45 cycles of 95ºC for 15 seconds, and 60ºC for 60 seconds.

Genotyping Assay

Because the duplex assay is not genotype specific for *T. orientalis*, any sample that was positive according to that assay was additionally genotyped. All duplex assay and genotyping assay results were recorded in our sample database.

Analysis

The sample database is an excel file containing pertinent cattle information, *T. orientalis* status with genotype information available, and *A. marginale* status. A filter was applied to specifically analyze those animals that returned a positive result on the duplex assay (marginal animals were excluded from this study). Within this sample, an additional filter was applied to rule in *T. orientalis* positive individuals. Animals that returned a positive for both *A. marginale* and *T. orientalis* were included in this study. The results from the genotype assays were then examined.

Results

57 animals were positive for both *A. marginale* and *T. orientalis*. Of those animals, only 43 had genotypes reported; these animals were included in the final study. 16 were positive for Chitose, and 27 were positive for Ikeda.

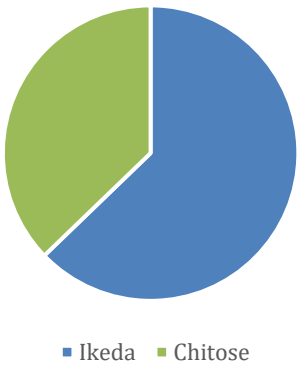

Figure 1. The proportion of animals affected by a given genotype of *T. orientalis* in those animals that are co-infected with *A. marginale*. 27 of 43 animals were positive for Ikeda (62.8%), and 16 of 43 were positive for Chitose (37.2%)

Conclusion

The initial data in our database indicate that most co-infections involve Ikeda-positive animals. This is not entirely surprising; of 566 genotyped *Theileria*-positive entries in our databank, 527 of them are Ikeda positive, compared to 38 Chitose positive (1 is Buffeli positive). Assuming no external factors impact the co-infection rate of *Anaplasma* and *Theileria*, we can reasonably expect the majority of dual infected animals to be positive for Ikeda compared to Chitose. That said, it is interesting that of all of the Chitose-positive samples in our databank, nearly half of them are additionally *Anaplasma* positive. This opens several potential areas of investigation:

1. Are *Anaplasma*-positive animals more likely to become co-infected with Chitose, or are Chitose-positive animals more likely to become co-infected with *Anaplasma?*

2. Is this due to synergistic effects of the microbe, impairment of the immune system by one organism or the other?

3. Is this due to inherent tick vector factors not yet elucidated (including topographic distribution)?

Conclusions and Future Directions

There is still a lot to learn about the pathogenesis of the mechanisms of anemia due to theileriosis in Virginia, and much to learn about the disease dynamics throughout the United States as a whole. The tick is likely to remain a vector throughout the state, and theileriosis is likely to become endemic. Considering the lack of current FDA approved treatment, mitigating the impacts on producer income, industry economics, and animal health will rely on controlling the tick vector. Much of this is going to rely on preventing tick parasitism, as the biology of this tick is likely to allow for rapid establishment of endemic populations in the US. Tick control will be additionally beneficial not just in the prevention of theileriosis, but in the prevention of anaplasmosis, as well. Although the tick vectors for these agents differ, tick control is broad, and identifying a method that prevents parasitism by multiple tick species will allow for improved cattle health throughout the state. However, these interventions need to be pursued with care, as off-target anti-parasitic resistance is of great concern to all components of the One Health triad: animal, plant, and human health. In the meantime, appropriate identification of the etiology of IBA on a case-by-case basis remains key for appropriate use of antibiotics in our food animal species. In an era of increased awareness of antimicrobial resistance and increasingly rigorous antimicrobial stewardship efforts, this is an important consideration.

The work completed as part of this dissertation supports the hypothesis that *T. orientalis* Ikeda present in Virginia, and can co-occur with *A. marginale*. Transmission studies by our collaborators in Washington further indicate that *T. orientalis* can be transmitted by *H. longicornis* in the United States, and additionally suggest that *T. orientalis* Ikeda alone can cause anemia. To help bolster this argument, and to further elucidate pathogenesis and potentially identify molecular targets for therapy, studies examining the molecular basis for disease are warranted. A reasonable starting point would be examining whether the major piroplasm surface protein has additional impact once inside the red blood cell, or if molecular damage to erythrocytes occurs due to the physical impact of replicating merozoites.

Our work additionally demonstrates that *T. orientalis* Ikeda and *A. marginale* are capable of co-infecting a single animal. This is unique to the United States. Other countries with *T. orientalis* Ikeda and *A. marginale* have these diseases separated geographically within the country, as a result of the tick vectors being geographically distant. Additional examination of the clinical impact of co-infection is a worthwhile investigation. Comparing PCV and/or HCT and conducting full statistical analysis to elucidate significantly significant impact of *T. orientalis* Ikeda, *T. orientalis* Chitose, *A. marginale*, and various co-infection status thereof would be useful knowledge for the medical community to possess. To date, all *T. orientalis* positive clinical submissions have been Ikeda positive, suggesting a greater clinical impact of Ikeda genotype in VA. Further investigation of the relationship between *Anaplasma* and Chitose is especially warranted, given that a substantial percentage of our Chitose-positive animals are additionally infected with *A. marginale*.

Appendices

Appendix A: Bayesian Mixture Models

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Bayesian mixture models are a statistical method that can be applied when a gold standard diagnostic test is not available. These tests utilize a host of prior information to build models of statistical likelihood, and then compare collected data against those models. The strengths of this particular type of analysis are many, and are arguably more robust than many of the analyses used in veterinary diagnostic medicine to this point in time. This analysis is based on biologically plausible principles; specifically, that the log distribution of the measured indicator (such as Ct values for rtPCR) is Gaussian in both disease positive and disease negative populations. Rather than assessing uncertainty – a significant impactor on clinical decision-making – after applying a cut-off point, uncertainty can be assessed at the level of parameter estimates. Relatedly, the outcomes are more informative than in diagnostic tests with hard cut-offs, because the outcomes of a mixture model are based on the probabilities of positivity. Finally – and importantly – covariates (such as geographic location, sex, animal use, age, reproduction status, etc.) can be easily incorporated into the model, and many data sources can be integrated into a single model. (Swart, et al 2021).

As with most inferential statistical tests, the strength of the data depends on the degrees of freedom; as such, utilizing multiple assays and comparing multiple populations improves the inferential ability. Our database includes multiple populations. We have access to information from VA Department of Correction cattle herds, which we can compare to non-VDOC, privately owned animals. We have beef cattle and dairy cattle. With GIS information collected at the time of sampling through our convenience sampling efforts with the VA Department of Agriculture and Consumer Services, we also have the ability to sort our data into geographical populations throughout the state (for example, cattle from the piedmont region compared to cattle from the tidewater region, or cattle from the eastern part of the state compared to cattle from the western part of the state, etc.).

These models are particularly useful in veterinary epidemiological studies with emerging infectious disease, where a gold standard test does not yet exist. By utilizing prior information collected by researchers in other

countries, we can infer the general prevalence of *T. orientalis* Ikeda, and use that information to build models against which to compare our collected data.

There is a growing push to utilize Bayesian mixture models in data analysis in veterinary epidemiological studies. There is, unfortunately, a higher barrier to incorporation of these assays, as they are more complicated and mathematically involved than existing analyses. While we did not run one in the inclusion of this study (largely for these reasons), we highly recommend developing the relationship with in-house statistical services, and developing protocols for the use of these analyses in the future.

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