

1 Co-Infection of cattle in Virginia with *Theileria orientalis ikeda* genotype and *Anaplasma*
2 *marginale*

3 **Vanessa J. Oakes, S. Michelle Todd, Amanda A. Carbonello, Pawel Michalak, Kevin K.**
4 **Lahmers¹**

5 Department of Biomedical Sciences and Pathobiology (Oakes, Lahmers); Virginia Tech Animal
6 Laboratory Services (Todd, Carbonello, Lahmers), Virginia-Maryland College of Veterinary
7 Medicine, Blacksburg, VA; Edward Via College of Osteopathic Medicine, Monroe, LA
8 (Michalak); Institute of Evolution, University of Haifa, Israel (Michalak).

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10 ¹Corresponding author: Kevin K. Lahmers, Department of Biomedical Sciences and
11 Pathobiology, Virginia-Maryland College of Veterinary Medicine, 205 Duck Pond Dr.,
12 Blacksburg, VA 24061. klahmers@vt.edu

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14 Running head: *Theileria orientalis* and *Anaplasma marginale* co-infection.

15 **Abstract**

16 *Theileria orientalis ikeda* is a newly identified agent of bovine infectious anemia in the
17 United States. Although it is transmitted by separate tick hosts than *Anaplasma marginale* – a
18 bacterial etiology of bovine infectious anemia –the geographic distributions of these two
19 infectious organisms overlap, with co-infection reported in some cattle. Only anaplasmosis has
20 approved effective treatment in the United States. To provide rapid diagnostic information for
21 producers with anemic animals, we developed a duplex qPCR for *A. marginale* and *T. orientalis*.
22 With a cut-off of 38 cycles, the duplex assay has a sensitivity of 96.97% and a specificity of
23 100% for *A. marginale*; with a cut-off of 45 cycles, the duplex assay has a sensitivity and a
24 specificity of 100% for *T. orientalis*. In addition to providing a tool for improved clinical
25 decision-making for veterinarians and producers, this qPCR facilitates the study of co-infection
26 rate of cattle in Virginia. Of 1,359 blood samples analyzed, 174 were positive for the presence of
27 *T. orientalis*, 125 were positive for the presence of *A. marginale*, and 12 samples were positive
28 for both *T. orientalis* and *A. marginale*. This indicated that co-infection of both of these
29 etiologies of bovine infectious anemia does occur within the state of Virginia. It is likely that this
30 pattern of infection will be seen in regions where *T. orientalis* and *A. marginale* are endemic,
31 despite the difference in tick vectors.

32 **Key words:** *Anaplasma marginale*, bovine infectious anemia, *Theileria orientalis ikeda*

33 **Introduction**

34 The *Theileria orientalis* complex describes several genotypes of a species of non-
35 proliferative, theilerial hemoprotozoan. Within the complex, the *ikeda* and *chitose* genotypes (*T.*
36 *orientalis ikeda* and *T. orientalis chitose*, respectively) are capable of causing disease. *T.*
37 *orientalis ikeda* in particular is increasingly implicated as a causative agent of bovine infectious

38 anemia in the United States, a vector-borne disease characterized by hemolytic anemia, icterus,
39 general malaise, ill thrift, and sporadic abortions. Although rarely fatal, affected animals are
40 often poorly producing; consequently, this is a disease of economic importance in the countries
41 in which it is found.⁹ *T. orientalis* is most effectively transmitted by the Ixodidae tick
42 *Haemaphysalis longicornis*,⁸ a tick which has recently been discovered in several states along the
43 US Eastern seaboard.¹³ *T. orientalis ikeda* has recently been identified in cattle in Virginia,
44 affecting animals that were also parasitized with *H. longicornis* ticks;¹⁰ this tick has been
45 confirmed as a competent vector for the Virginia *T. orientalis ikeda*.⁷ The animals that were
46 clinically affected in this outbreak presented with the typical signs of anemia, icterus, and
47 general malaise. In Virginia, this clinical presentation is identical to the blood infection caused
48 by the bacterium *Anaplasma marginale*. *Haemaphysalis longicornis* is not capable of
49 transmitting *A. marginale*,⁵ and in many other parts of the world, the geographic distribution of
50 *T. orientalis ikeda* and *A. marginale* do not overlap. In Virginia, however, the initial outbreak of
51 *T. orientalis ikeda* occurred in areas where anaplasmosis has been diagnosed historically,
52 suggesting an overlap in geographic distribution in this region. Further, the predicted range of *H.*
53 *longicornis* based on modeling suggests its presence in areas of the country where anaplasmosis
54 is routine.¹² An animal included in the initial outbreak study in Virginia was positive for both *T.*
55 *orientalis ikeda* and *A. marginale* by conventional PCR and Sanger sequencing¹⁰. As a bacterial
56 infection, anaplasmosis is a treatable condition using oxytetracycline, a cost-effective drug
57 approved for use in food animals. Theileriosis is markedly more difficult to treat, and no
58 effective drugs are approved for use in food animals in the United States, although
59 Buparvaquone in Australia has been effective.⁴ Therefore, making an early distinction between
60 anaplasmosis and theileriosis is critical for immediate and effective clinical decision-making that

61 has welfare, productivity, and economic implications. It is important that the tools used to assist
62 in this process are cost-effective, to be of use to producers.

63 In response, we developed a duplex real-time PCR assay that is sensitive for both *A.*
64 *marginale* and *T. orientalis*. We further identify the genotype of *T. orientalis* for *Theileria*-
65 positive samples with an additional multiplex qPCR genotype assay (included in the cost to the
66 producer). Previous multiplex assays examining *Theileria* spp., *Babesia* spp. Protozoan, and
67 *Anaplasma* spp. bacteria have been developed in ruminants,^{1,6} so we sought to fill the niche for
68 cattle.

69 **Materials and Methods**

70 Blood

71 Whole blood samples were submitted from client-owned cattle across the state of
72 Virginia. Animals represented clinical submissions by private veterinarians, as well as herds
73 owned and maintained by the Virginia Department of Corrections (VADOC), and as part of an
74 ongoing, random sampling surveillance effort of animals sent to auction by the Virginia
75 Department of Agriculture and Consumer Services (VDACS). Whole blood was collected in
76 purple-top BD Vacutainer® blood collection tubes containing EDTA anticoagulant (Becton,
77 Dickinson and Company). Except for animals submitted by referring veterinarians as part of a
78 routine clinical diagnostic workup, all animals were randomly sampled, and the presence and
79 degree of clinical signs were unknown. In total, 1,359 blood samples were available for
80 evaluation.

81 DNA Extraction

82 DNA was extracted from EDTA anticoagulated blood with the DNeasy® Blood and
83 Tissue Kit (Qiagen) following the manufacturer's protocol with a few modifications.¹⁰ The initial

84 blood volume for DNA extraction was 100 μ L, and Applied BiosystemsTM VetMaxTM XenoTM
85 Internal Positive Control DNA (ThermoFisher Scientific) was added to the lysis buffer (Buffer
86 AL) at 20,000 copies per sample. DNA was eluted twice in 50 μ L of Buffer AE pre-heated to
87 56°C for a total elution volume of 100 μ L.

88 Duplex qPCR

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

94 Amplification for the msp1b and MPSP were accomplished in the same reaction. The duplex
95 qPCR reaction consisted of Applied Biosystems[®] TaqManTM Environmental Master Mix 2.0
96 (ThermoFisher Scientific), 300nM of each *T. orientalis* forward and reverse primers, 600nM of
97 each *A. marginale* forward and reverse primers, 100nM of *T. orientalis* Universal probe, 200nM
98 of *A. marginale* probe, 0.8 μ L of XenoTM VICTM Primer-Probe Mix (ThermoFisher Scientific),
99 and 2 μ L of DNA template in a 20 μ L reaction. The primers and probes for *T. orientalis* and *A.*
100 *marginale* have been previously published.^{2,3} Fluorophores and quenchers were altered from the
101 published probes in order to allow for appropriate multiplexing. (Table 1) The probes utilize
102 three separate fluorescent tags - FAMTM for *A. marginale*, NEDTM for *T. orientalis*, and VIC[®] for
103 the internal positive control. Amplification was completed in an Applied BiosystemsTM 7500
104 Fast Real-time PCR System (ThermoFisher) using standard cycling and the following run
105 method: (95°C^{10:00}) (95°C^{0:15}, 60°C^{1:00})_{x45}. Reactions were completed in Applied Biosystems[®]
106 MicroAmp[®] Fast 8-Tube Strips with Applied Biosystems[®] MicroAmp[®] Optical 8-Cap Strips

107 (ThermoFisher Scientific). A sample lacking DNA template was included as a negative control
108 with each run. A *T. orientalis* known positive sample, an *A. marginale* known positive sample,
109 and 2,000 copies of VetMax™ Xeno™ Internal Positive Control DNA (ThermoFisher Scientific)
110 were included in separate reactions with each run to serve as positive controls for the respective
111 fluorophore channels.

112 **Validation: Limit of Detection**

113 To determine the limit of detection for *T. orientalis*, three separate identical qPCR runs
114 were completed per the previously described protocol. Eight, 10-fold serial dilutions of
115 gBlocks™ Gene Fragments (Integrated DNA Technologies) with the universal MPSP gene
116 sequence capable of detecting each of the genotypes *ikeda*, *chitose* and *buffeli* were used. The
117 limit of detection was determined as the lowest dilution at which samples on all three plates were
118 positive, and a limit of detection was determined for each of the three *T. orientalis* genotypes of
119 interest. Similarly, the limit of detection was determined for *A. marginale* using serial dilutions
120 of an *A. marginale*-positive blood sample confirmed positive by external qPCR and shown to
121 have 1.22% infected RBC.

122 **Validation: Repeatability**

123 Inter-assay and intra-assay repeatability studies were conducted. Intra-assay repeatability
124 tests the precision of a single run by examining the high, medium, and low concentrations of
125 reference DNA in quintuplicate in one run on one day. In this assay, the average Ct is compared
126 to the standard deviation of the five replicates of a given concentration. Inter-assay repeatability
127 tests high, medium, and low concentrations of reference DNA in quintuplicate on each of six
128 consecutive days. In this assay, the average Ct values of a given concentration are compared

129 across the 6 consecutive plates. All repetitions were performed on the same Applied Biosystems®
130 7500 Fast Real-Time PCR system (ThermoFisher Scientific).

131 Validation: Sensitivity and Specificity

132 The sensitivity and specificity of the *T. orientalis* and *A. marginale* targets within the
133 duplex assay were determined with receiver operating characteristic (ROC) statistics. Our
134 genotype-specific *T. orientalis* qPCR described below was used as the gold-standard test, against
135 which the duplex qPCR results were compared. For *A. marginale*, qPCR was completed either at
136 the United States Department of Agriculture's Agricultural Research Service, Animal Disease
137 Research Unit (USDA-ARS-ADRU) (Pullman, WA, USA) or at the Washington Animal Disease
138 Diagnostic Laboratory (WADDL) (Washington State University, Pullman, WA, USA) as the
139 gold-standard test.

140 Genotyping qPCR

141 A second multiplex assay that utilizes distinct probes specific for the MPSP of three of
142 the major *T. orientalis* genotypes - *chitose*, *ikeda*, and *buffeli* further characterizes the genotype.
143 The forward and reverse primers are the same as those used for *T. orientalis* in the duplex qPCR
144 protocol above, but with the use of a distinct set of probes to distinguish between the genotypes.²
145 (Table 2)

146 DNA extracted from whole blood samples that were positive for *T. orientalis* on the
147 duplex qPCR were used for the genotype multiplex qPCR. The 20 µL reactions consisted of
148 Applied Biosystems® TaqMan™ Environmental Master Mix 2.0 (ThermoFisher Scientific),
149 300nM each *T. orientalis* forward and reverse primers, 250nM of Ikeda probe, 100nM of Chitose
150 A probe, 150nM Chitose B probe, 100nM Buffeli probe, and 2µL of DNA (Table 2). The
151 instrumentation, run method, and plastics were the same as for the duplex qPCR Assay. A

152 sample lacking DNA template was included with each run as a negative control. Positive
153 controls for each of the three genotypes were included in each run.

154 **Chitose Conventional PCR and Sanger**

155 DNA that tested positive for the *chitose* genotype of *T. orientalis* in the duplex qPCR
156 were subjected to two rounds of conventional PCR (cPCR) targeting the MPSP and SSU genes
157 of *T. orientalis*. The reaction for the first round of cPCR consisted of Invitrogen™ Platinum®
158 PCR SuperMix High Fidelity (Thermo Fisher Scientific), 400nM each of sense and anti-sense
159 primers, and 10.5µl DNA in a 25µl reaction volume. Primers (Integrated DNA Technologies)
160 for MPSP were 5'-CTTGCGCTAGGATACTTCCT-3' (sense) and 5'-
161 ACGGCAAGTGGTGAGAACT-3' (anti-sense).¹¹ Primers for SSU were 5'-
162 ATTGGAGGGCAAGTCTGGT-3' (sense) and 5'- CTCTCGGCCAAGGATAAACTCG-3'
163 (anti-sense).⁹ The PCR program was as follows: 95°C 2:00 (95°C 0:15, 57°C 0:30, 72°C 1:00)_{x29}, 72°C
164 7:00, 12°C 0:00. A second round of cPCR was carried out using the amplicons from the first round
165 as template using the same reaction components and PCR program. Second round amplicons
166 were electrophoresed on a 1% TBE-agarose:EtBr gel and visualized under UV light. DNA
167 bands were purified from the gel using the QIAquick® Gel Extraction Kit (Qiagen) and
168 submitted to the Fralin Life Sciences Institute, Genomic Sequencing Center for Sanger
169 sequencing.

170 **Results**

171 **Duplex qPCR**

172 1,359 individual DNA samples were available for qPCR testing. 186 (13.7%) were
173 positive for the presence of *T. orientalis*, and 137 (10.1%) were positive for the presence of *A.*

174 *marginale*. Included in these numbers are 12 samples (0.88%) that were positive for both *T.*
175 *orientalis* and *A. marginale*.

176 Limit of Detection

177 The limit of detection for *T. orientalis* genotypes *ikeda* and *buffeli* were 1×10^8 picomoles.
178 For the *chitose* genotype, the limit of detection was 1×10^9 picomoles. *A. marginale* was detected
179 according to dilution rate, given the nature of the known positive sample. The limit of detection
180 of *A. marginale* was a 10^{-5} dilution.

181 Repeatability

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

186 Sensitivity and Specificity

187 For *A. marginale*, the assay sensitivity was 96.97% and the specificity was 100% when a
188 cut-off of 38 cycles was applied. (Table 4, Table 5) For *T. orientalis*, both the sensitivity and the
189 specificity were 100% when a cut-off of 45 cycles was applied (the final cycle of the qPCR
190 program). (Table 6, Table 7) The gold standard utilized for *A. marginale* was an external qPCR,
191 run either at the United States Department of Agriculture's Agricultural Research Service,
192 Animal Disease Research Unit (Pullman, WA, USA), or at the Washington Animal Disease
193 Diagnostic Laboratory (Washington State University, Pullman, WA, USA).

194 The gold standard utilized for *T. orientalis* was the *T. orientalis* genotype qPCR
195 developed by the Virginia Tech Animal Laboratory Services (Virginia-Maryland College of
196 Veterinary Medicine), described below.

197 **Genotyping**

198 Because of the use of a universal probe for the MPSP gene of *T. orientalis*, an additional
199 genotyping assay was required for those samples that tested positive for *T. orientalis* in the
200 duplex assay. The genotype multiplex assay was run using extracted DNA, applying the same *T.*
201 *orientalis* forward and reverse primers, but utilizing genotype-specific MPSP probes. Samples of
202 known genotype were included as positive controls. Out of 186 *T. orientalis*-positive samples
203 from the duplex PCR, 159 of them (85.5%) were consistent with the *ikeda* genotype, 21 were
204 consistent with the *chitose* genotype, and the remaining 6 (3.2%) were undetermined. None were
205 consistent with *buffeli*. There were no mixed infections with multiple genotypes.

206

207 **Discussion**

208 *T. orientalis ikeda* genotype/genotype 2 is an emerging pathogen within Virginia, and is
209 increasingly implicated globally as an etiologic agent of bovine infectious anemia. The clinical
210 signs of theileriosis and anaplasmosis are identical, consisting of anemia, icterus, poor-doing,
211 decreased productivity, and occasional, sporadic abortions. However, in the United States,
212 oxytetracycline is an approved and effective drug for the treatment of *A. marginale*, whereas
213 there exists no approved drug for the treatment of *T. orientalis ikeda*. Consequently, there is a
214 need for an affordable, effective diagnostic test capable of differentiating between theileriosis
215 and anaplasmosis.

216 Theileriosis and anaplasmosis overlap within Virginia. This is most likely attributable to
217 a mixed tick infestation in the region. In the United States, *Dermacentor* spp. ticks are the
218 vectors for *A. marginale*.¹⁴ While *Dermacentor nuttali* ticks have been shown to transmit some
219 genotypes of *T. orientalis* in some parts of the world,¹⁵ there is no evidence that they are

220 competent vectors for the *ikeda* genotype specifically. Similarly, *H. longicornis*, the primary tick
221 vector of *T. orientalis* *ikeda* globally, is not a competent vector for *A. marginale*.¹⁵

222 Also of interest in this current study is the presence of *T. orientalis* *chitose* in some of the
223 submitted clinical samples, without co-infection with *ikeda* or *A. marginale*. Like *ikeda*, this is a
224 genotype of emerging importance, as it is increasingly implicated as a non-transforming,
225 pathogenic disease of cattle.⁹ Current understanding on the *T. orientalis* complex suggest *ikeda*
226 and *chitose* have the potential to be pathogenic; *buffeli* is endemic to the United States and
227 elsewhere in the world, and is an incidental, non-pathogenic finding.^{9,10} This difference in
228 genotype pathogenicity is the rationale behind the genotyping component of the assay described
229 in this paper.

230 The high sensitivity for *A. marginale* and *T. orientalis* makes this duplex qPCR assay a
231 useful screening tool for producers in Virginia and in other localities with co-localization of
232 anaplasmosis and theileriosis. Further investigation into the geographical distribution of *A.*
233 *marginale* and pathogenic genotypes of *T. orientalis*, including *chitose*, as a probable agent of
234 disease is warranted.

235

236 **Acknowledgements**

237 The authors would like to extend immense gratitude to the veterinarians and producers involved
238 in ongoing surveillance efforts.

239

240 **Declaration of conflicting interests**

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

243

244 **Funding**

245 Portions of this study were funded by the Virginia College of Osteopathic Medicine/Virginia-
246 Maryland College of Veterinary Medicine Center of One Health Seed grant program.

247

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282 **Tables and Figures**

283 **Table 1.** Primer and probe sequences used for *T. orientalis* and *A. marginale* duplex PCR assay.

Target	Sequence
<i>T. orientalis</i>	
Forward primer	5'-GCA AAC AAG GAT TTG CAC GC-3'
Reverse primer	5'-TGT GAG ACT CAA TGC GCC TAG A-3'
Universal probe	5' NED-TCG ACA AGT TCT CAC CAC-MGB-NFQ 3'
<i>Anaplasma marginale</i>	
Forward primer	5'-TTG GCA AGG CAG CAG CTT-3'
Reverse primer	5'-TTC CGC GAG CAT GTG CAT-3'

284

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

Probe Target	Sequence
<i>T. orientalis ikeda</i>	5' VIC-CAT GGA CAG TGC TTG GC-MGB-NFQ 3'
<i>T. orientalis chitose A</i>	5' NED-TCC TCA GCG CTG TTC T-MGB-NFQ 3'
<i>T. orientalis chitose B</i>	5' NED- TCC TCG GCG CTG TTC T-MGB-NFQ 3'
<i>T. orientalis buffeli</i>	5' FAM- CTC CTT TGC AGT ATT CTT CTA TCT C-QSY 3'

286

287 **Table 3.** Comparison of One-Way ANOVA results following the repeatability assay.

Target	Concentration	One-Way ANOVA	Variance
<i>A. marginale</i>	Undiluted	<0.0001	Yes
	10 ⁻²	0.0025	Yes
	10 ⁻⁴	0.0007	Yes
<i>T. orientalis buffeli</i>	Undiluted	0.7828	No
	10 ⁻¹	0.9730	No
	10 ⁻³	0.5484	No
<i>T. orientalis chitose</i>	Undiluted	0.2784	No
	10 ⁻¹	0.4041	No
	10 ⁻²	0.4091	No
<i>T. orientalis ikeda</i>	Undiluted	0.1583	No
	10 ⁻¹	0.7370	No
	10 ⁻²	0.1495	No

288

289 **Table 4.** A comparison of the sensitivity and specificity values for *A. marginale* when compared
290 to an external qPCR gold standard.

Cutpoint (Ct)	Sensitivity	Specificity
45.00	1.0000	0.6818
40.00	0.9697	0.7955
39.00	0.9697	0.9733
38.00	0.9697	1.0000
37.00	0.9697	1.0000
36.00	0.9394	1.0000

291

292 **Table 5.** An overview of the positive and negative cases for *A. marginale* when compared to an
293 external qPCR gold standard.

294

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

295

296 **Table 6.** A comparison of the sensitivity and specificity values for *T. orientalis* when compared
297 to a validated qPCR gold standard.

Cutpoint (Ct)	Sensitivity	Specificity
45.00	1.0000	1.0000
40.00	1.0000	1.0000
39.00	1.0000	1.0000
39.00	0.9697	1.0000
37.00	0.9388	1.0000
36.00	0.9	1.0000

298

299 **Table 7.** An overview of the positive and negative cases for *T. orientalis* when compared to a
300 validated qPCR gold standard.

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

301