International Journal for Parasitology xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Discovery of a novel species, *Theileria haneyi* n. sp., infective to equids, highlights exceptional genomic diversity within the genus *Theileria*: implications for apicomplexan parasite surveillance ☆

Donald P. Knowles [a,b,c,d,1,2,*], Lowell S. Kappmeyer [a,1,2], Darrell Haney [e,3], David R. Herndon [a,3], Lindsay M. Fry [a,b,3], James B. Munro [f,3], Kelly Sears [b,g,3], Massaro W. Ueti [a,b,c,3], Lauren N. Wise [h,3], Marta Silva [b,3], David A. Schneider [a,b,3], Juanita Grause [i,3], Stephen N. White [a,b,3], Kyle Tretina [f,3], Richard P. Bishop [b,c,3], David O. Odongo [j,3], Angela M. Pelzel-McCluskey [k,3], Glen A. Scoles [a,3] Robert H. Mealey [b,3], Joana C. Silva [f,l,*]

[a] *Animal Disease Research Unit, Agricultural Research Service, United States Department of Agriculture, Pullman, WA, USA*
[b] *Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA, USA*
[c] *The Paul G. Allen School for Global Animal Health, Washington State University, Pullman, WA, USA*
[d] *Department of Comparative Medicine, University of Washington School of Medicine, Seattle, WA, USA*
[e] *Animal and Plant Health Inspection Service, United States Department of Agriculture, Eagle Pass, TX, USA*
[f] *Institute for Genome Sciences, University of Maryland School of Medicine, Baltimore, MD, USA*
[g] *Veterinary Clinical Sciences, Washington State University, Pullman, WA, USA*
[h] *Department of Large Animal Medicine and Surgery, Physiology and Pharmacology, St. George's University School of Veterinary Medicine True Blue, St. George's, Grenada*
[i] *Animal and Plant Health Inspection Service, National Veterinary Services Laboratories, United States Department of Agriculture, Ames, IA, USA*
[j] *Department of Zoology, Nairobi University, Nairobi, Kenya*
[k] *Animal and Plant Health Inspection Service, Surveillance, Preparedness and Response Services, United States Department of Agriculture, Fort Collins, CO, USA*
[l] *Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD, USA*

ARTICLE INFO

Article history:
Received 11 September 2017
Received in revised form 12 March 2018
Accepted 22 March 2018
Available online xxxx

Keywords:
Theileria
Protein sequence divergence
Phylogenetic analysis
18S rDNA
Equi merozoite antigen superfamily
Equids

ABSTRACT

A novel apicomplexan parasite was serendipitously discovered in horses at the United States – Mexico border. Phylogenetic analysis based on 18S rDNA showed the erythrocyte-infective parasite to be related to, but distinct from, *Theileria* spp. in Africa, the most similar taxa being *Theileria* spp. from waterbuck and mountain zebra. The degree of sequence variability observed at the 18S rDNA locus also suggests the likely existence of additional cryptic species. Among described species, the genome of this novel equid *Theileria* parasite is most similar to that of *Theileria equi*, also a pathogen of horses. The estimated divergence time between the new *Theileria* sp. and *T. equi*, based on genomic sequence data, is greater than 33 million years. Average protein sequence divergence between them, at 23%, is greater than that of *Theileria parva* and *Theileria annulata* proteins, which is 18%. The latter two represent highly virulent *Theileria* spp. of domestic cattle, as well as of African and Asian wild buffalo, respectively, which differ markedly in pathology, host cell tropism, tick vector and geographical distribution. The extent of genome-wide sequence divergence, as well as significant morphological differences, relative to *T. equi* justify the classification of *Theileria* sp. as a new taxon. Despite the overall genomic divergence, the nine member equi merozoite antigen (EMA) superfamily, previously found as a multigene family only in *T. equi*, is also present in the novel parasite. Practically, significant sequence divergence in antigenic loci resulted in this undescribed *Theileria* sp. not being detectable using currently available diagnostic tests. Discovery of this novel species infective to equids highlights exceptional diversity within the genus *Theileria*, a finding with serious implications for apicomplexan parasite surveillance.

☆ *Note:* Nucleotide sequence data reported in this paper are available in GenBank under accession numbers KU647704–KU647710.

* Corresponding authors at: 3003 ADBF, Washington State University, Pullman, WA 99164-6630, USA. Fax: +1 509 335 8328 (D.P. Knowles). BioPark II, 645, University of Maryland School of Medicine, 655 W. Baltimore Street, Baltimore, MD 21201, USA. Fax: +1 410 706 6756 (J.C. Silva).
E-mail addresses: dknowles@wsu.edu (D.P. Knowles), JMunro@som.umaryland.edu (J.B. Munro), jcsilva@som.umaryland.edu (J.C. Silva).
[1] Equal first authors.
[2] These authors contributed equally.
[3] These authors contributed equally to this work.

https://doi.org/10.1016/j.ijpara.2018.03.010
0020-7519/Published by Elsevier Ltd on behalf of Australian Society for Parasitology.

Please cite this article in press as: Knowles, D.P., et al. Discovery of a novel species, *Theileria haneyi* n. sp., infective to equids, highlights exceptional genomic diversity within the genus *Theileria*: implications for apicomplexan parasite surveillance. Int. J. Parasitol. (2018), https://doi.org/10.1016/j.ijpara.2018.03.010

1. Introduction

Hemoparasites that cause life-long infection, such as those in the phylum Apicomplexa, are a major challenge for public and livestock health. Notorious examples include the arthropod-borne apicomplexan taxa *Plasmodium* and *Babesia*, for which infection in humans and cattle, respectively, result in malaria and bovine babesiosis, and theileriosis, which infects ruminants and equids (Kappmeyer et al., 2012), in addition to many other vertebrates. Discerning how apicomplexan protozoa emerge and persist in host populations is critical for controlling disease and transmission. Propagation of these organisms during long-term persistent infection results in genomic evolution that potentially impacts virulence, host range and reduced detectability (Hawley et al., 2013). The diversity of apicomplexans, especially *Theileria*, in wildlife is exceptional (Githaka et al., 2014; Mans et al., 2015). Vector-mediated movement of these organisms from wildlife to livestock can result in disease outbreaks that are not identifiable using current diagnostic assays. Data presented here document the molecular biology, genomic composition and phylogenetic placement of a newly discovered *Theileria* pathogen that has serious implications for diagnosis of *Theileria* infections in equids.

Equine piroplasmosis (theileriosis), caused by *Theileria equi*, is a tick-borne infection of equids for which control by vaccination is not currently available. Serological and nucleic acid-based diagnostic testing, followed by treatment of infected animals with imidocarb dipropionate (Ueti et al., 2012), is the method routinely used for transmission and disease control in non-endemic countries. Animal reservoirs for transmission include all equid species (Wise et al., 2013). Infection with *T. equi* can lead to a life-threatening anemia in the acute phase, and results in persistent infection in the chronic phase (Wise et al., 2013). Horses that survive the acute phase will be infected for life, with a parasitemia between 10^3 and 10^6 merozoites per mL of blood (Ueti et al., 2005), and thus serve as a reservoir for tick transmission. Since *Theileria* is not transmitted transovarially by the tick, ticks are not considered a generational reservoir. Although the United States (U.S.) is currently considered free of equine *Theileria* infection, recent detection of 20 infected horses in Florida, U.S. (Short et al., 2012) and 413 infected horses elsewhere, mostly in Texas, U.S. (Scoles et al., 2011), has increased awareness of the potential transmission and disease threats posed by asymptomatic *Theileria* infections (Ueti et al., 2005; Wise et al., 2014).

A stray horse, restrained at the U.S. – Mexico border, near Eagle Pass, Texas, was negative for *T. equi* by PCR, but borderline positive using a competitive ELISA (cELISA) (Knowles et al., 1991b). A hemoprotozoan parasite was recovered by whole blood transfer to a naïve splenectomized horse, and the genome sequenced. Based on parasite morphology and intra-erythrocytic replication, the parasite was identified as a species of *Theileria*. The implications of these data are discussed in the context of disease control of vector-borne apicomplexan parasites in general, but with specific reference to equine *Theileria*.

2. Materials and methods

2.1. Ethics statement

All animal experiments were approved by the University of Idaho, U.S., Institutional Animal Care and Use Committee (IACUC), protocol numbers 2016–18, 2016–28 and 2016–29. The animal care and use protocol adhered to the Animal Welfare Act set forth under the U.S. Regulations and Standards in the Title 9 Code of Federal Regulations (CFR), Chapter 1, Subchapter A – Animal Welfare.

2.2. Horses (*Equus ferus caballus*)

This study utilized horses of various breeds and ages. The following horses were instrumental in work presented here. Three horses (H14, H16915 and H21490) were from the region of Eagle Pass, Texas. Seven additional horses, namely H196(S), H208(S), H270, H296, H233, H248(S) and H301(S), where (S) stands for "splenectomized", were derived from a breeding herd located at the University of Idaho, under protocol number 2016–18. All whole blood transfers were carried out in citrate phosphate dextrose anticoagulant (Fenwal Inc., U.S.). H14 was the original source of blood. Splenectomized horse H208(S) received 120 mL of whole blood from H14. Two spleen-intact horses, H270 and H296, received 120 mL of whole blood from H16915 and H21490, respectively. Spleen-intact horse H233 received a 2 mL stabilate, with 12% parasitized erythrocytes (PPE), derived from H208(S). Stabilates were prepared as follows: whole blood was collected by jugular venipuncture into a vacuum flask containing 200 g of 5 mm glass beads and swirled until clotting occurred and blood defibrinated. Erythrocytes and plasma were transferred to conical centrifuge tubes and centrifuged at 1,400*g* for 10 min at 4 °C and plasma and buffy coat discarded. The erythrocyte pellet was washed four times in Puck's Saline G solution (Life Technologies, U.S.) using 1,400*g* for 10 min at 4 °C centrifugation. An equal volume of 20% polyvinylpyrrolidone in Puck's Saline G cryopreservative was added and aliquoted into cryogenic vials. Vials were frozen to −80 °C overnight and transferred to liquid nitrogen for permanent storage the next day. Splenectomized horses H248(S) and H301(S) received 120 mL of whole blood from H233. H196(S) was infected with *T. equi*™ (the reference *T. equi* Texas isolate) via intravenous inoculation with 120 mL of whole blood from horses maintained at the University of Idaho. Experimental horses received regular health checks from a licensed veterinarian. Prior to blood transfer, complete blood counts (CBCs) and serum chemistry panels were normal.

2.3. Comparative morphological analysis of asexual intra-erythrocytic forms of *T. equi*™ and parasite from H248(S)

A Nikon Upright Microscope Eclipse Ci, with calibrated scale, was used to measure parasite dimensions. Giemsa-stained blood slides were examined with a Nikon Plan Fluor at 100X/1.30 oil. The width and length of 40 individual masses from 10 Maltese crosses (Yokoyama et al., 2003), were measured for *T. equi*™ and the parasite from H248(S). The mean and standard deviation as well as the maximum width and length of individual Maltese cross masses were determined. The giemsa-stained blood slides were obtained from H248(S) and H196(S) infected with the new parasite or *T. equi*™, respectively (Scoles et al., 2011; Ueti et al., 2012). *Theileria equi*™ was chosen because it was obtained from the same region as the parasite derived from H248(S). Whole blood was collected from both H248(S) and H196(S) at 14 days p.i. Images were acquired using an Axio Imager M1 microscope (Carl Zeiss Microimaging, Thornwood, NY, USA) equipped with an LED bright field illuminator, Plan-Apochromat 63x/1.40 Oil M27 objective, AxioCam MRc5 digital camera, and AxioVision software (version 4.8.2.0; Carl Zeiss Microimaging, Thornwood, NY, USA). Images were exported in TIFF format and prepared for presentation using Photoshop Elements (version 5.0, Adobe Systems Inc., San Jose, CA, USA).

2.4. Genomic sequencing and preliminary genome annotation

Parasite genomic DNA (gDNA) was obtained from infected red blood cells of H248(S) and was used to generate a 3 Kbp Illumina

Please cite this article in press as: Knowles, D.P., et al. Discovery of a novel species, *Theileria haneyi* n. sp., infective to equids, highlights exceptional genomic diversity within the genus *Theileria*: implications for apicomplexan parasite surveillance. Int. J. Parasitol. (2018), https://doi.org/10.1016/j.ijpara.2018.03.010