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Molecular detection and characterization of *Anaplasma platys* and *Ehrlichia canis* in dogs from the Caribbean

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Abstract

Anaplasma platys is a tick-transmitted rickettsial pathogen, which is known to be the etiologic agent for cyclic thrombocytopenia in its primary canine host. Infections with this pathogen are also reported in cats, cattle and people. Similarly, *Ehrlichia canis* is another tick-borne rickettsial pathogen responsible for canine monocytic ehrlichiosis and is also reported to cause infections in people. We describe infections in dogs with these two pathogens on the Caribbean island of Grenada, West Indies by detection using molecular methods. We utilized a 16S rRNA gene-based PCR assay to detect both *Ehrlichia* and *Anaplasma* species by screening 155 canine blood samples from asymptomatic dogs. We found 18.7 % of the dogs to be positive for *A. platys* and 16.8 % for *E. canis*. Samples that tested positive for *A. platys* were further assessed by sequence analysis targeting 16S rRNA, 23S rRNA, citrate synthase *(gltA)* and heat shock protein *(groEL)* genes. Phylogenetic analysis revealed high correlation of *A. platys* 16S rRNA and *gltA* gene sequences with the geographic origins, while 23S rRNA and *groEL* gene sequences clustered independent of the geographic origins. This study represents an important step in defining the widespread distribution of active rickettsial infections in Caribbean dogs with no apparent clinical signs, thus

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CRediT authorship contribution statement

Andy Alhassan: Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Paidashe Hove:** Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Bhumika Sharma:** Resources, Investigation, Writing - review & editing. **Vanessa Matthew-Belmar:** Resources, Investigation. **Inga Karasek:** Resources, Investigation. **Marta Lanza-Perea:** Resources, Writing - review & editing. **Arend H. Werners:** Resources, Investigation. **Melinda J. Wilker- son:** Resources, Conceptualization, Formal analysis, Writing - review & editing, Supervision, Funding acquisition. **Roman R. Ganta:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Ethics statement

This study was performed in accordance with guidelines and approval given by the Institutional Animal Care and Use Committee (IACUC-17,006-R) at St. George's University, School of Veterinary Medicine.

Declaration of Competing Interest

The authors report no declarations of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2021.101727.

posing a high risk for canine health and to a lesser extent to humans, as most dogs in the Caribbean are free-roaming.

Keywords

Anaplasma platys; *Ehrlichia canis*; 16S rRNA; 23S rRNA; Citrate synthase (*gltA*) and heat shock protein *(groEL)*; Tick-borne diseases; PCR 1

1. Introduction

Anaplasma platys is an obligate intracellular, tick-borne rickettsial pathogen of the family *Anaplasmataceae* (Dumler et al., 2001), which infects platelets of its primary canine host (Sainz et al., 2015; Harvey et al., 1978). It is the causative agent of infectious canine cyclic thrombocytopenia, which was first described in 1978 by Harvey and colleagues in a dog infection from Florida, USA (Harvey et al., 1978). The biological vector for the pathogen is likely the brown dog tick, *Rhipicephalus sanguineus* sensu lato (Yuasa et al., 2017; Cicuttin et al., 2015; Ramos et al., 2014; Ybañez et al., 2012), although there is scant evidence for this (Gaunt et al., 2010; Simpson et al., 1991). One recent vector competence study, however, seems to provide strong evidence implicating *R. sanguineus* sensu stricto as a biological vector of *A. platys* (Snellgrove et al., 2020). This tick is also the vector for *Ehrlichia canis*, another *Anaplasmataceae* pathogen closely related to *A. platys*. Co-infections with *A. platys* and *E. canis* are commonly observed in dogs (Lanza-Perea et al., 2014; Yabsley et al., 2008).

In dogs, clinical signs with *A. platys* and *E. canis* infections may vary from asymptomatic to fever, weight loss, depression, weakness/lethargy, anorexia, lymphadenomegaly, splenomegaly, thrombocytopenia and weight loss (Mylonakis and Theodorou, 2017; Sainz et al., 2015; Harrus and Waner, 2011; Komnenou et al., 2007; Harrus et al., 1997a; Harvey et al., 1978). *Anaplasma platys* infections may result in petechiae, while *E. canis* may cause nose bleeds in some dog breeds. Ecchymoses and cyclical changes in platelet counts, possibly resulting from infection-associated drop in thrombocytes and host-induced immune countering, occur in dogs with *A. platys* infections. While it is likely that the canine immune system naturally controls the infection, the infection-associated immune suppression and fluctuating platelet counts may take a serious toll on the animal's health.

Recent molecular evidence from several studies suggests that *A. platys* has a global presence in *R. sanguineus* s.l. ticks and dogs (Latrofa et al., 2014) including in the USA (Diniz et al., 2010; Kordick et al., 1999), Argentina (Cicuttin et al., 2015; Eiras et al., 2013), Brazil (Soares et al., 2017), Grenada (Wilkerson et al., 2017; Lanza-Perea et al., 2014; Yabsley et al., 2008), St. Kitts (Loftis et al., 2013), Haiti (Starkey et al., 2016), Italy (Ramos et al., 2014), Portugal (Cardoso et al., 2010), Malaysia (Low et al., 2018), and Taiwan (Yuasa et al., 2017). *Anaplasma platys* is also reported to cause infections in domestic cattle in Algeria (Dahmani et al., 2015a), goats in China (Wei et al., 2020), and in cats (Qurollo et al., 2014; Lima et al., 2010) as well as in people in some South American and Caribbean countries (Arraga-Alvarado et al., 2014; Maggi et al., 2013), thus posing threat to wider host species, including humans. Similarly, *E. canis* infections are frequently reported in various host

species in parts of South America (Arroyave et al., 2020; Dumler, 2013; Eiras et al., 2013; Vieira et al., 2011; Vinasco et al., 2007), and Caribbean countries (Gondard et al., 2017; Kelly et al., 2017; Wilkerson et al., 2017; Loftis et al., 2013; Yabsley et al., 2008), as well as in Central America (Springer et al., 2019; Zhang et al., 2015; Romero et al., 2011). Dogs with persistent infections of either pathogen are highly susceptible to secondary infections, in addition to serving as sources of infection for the pathogen acquisition by naïve ticks and their subsequent transmission to naïve dogs as well as to people (Sainz et al., 2015). The brown dog tick, *R. sanguineus* s.l. has been implicated as the vector for these two pathogens as well as many other zoonotic rickettsial pathogens such as *Rickettsia conorii* and *Rickettsia rickettsii* (Dantas-Torres, 2010; Yabsley et al., 2008; Skotarczak, 2003; Lewis et al., 1977; Groves et al., 1975). This ectoparasite is also known to feed on people, although less frequently (Mentz et al., 2016; Dantas-Torres, 2010).

A vast amount of information can be gathered from performing systematic phylogenetic analyses of related species or strains of a species to make inferences on phylogeographic and evolutionary relationships (Martin, 2002). There is little or no information in literature that comprehensively elucidates the phylogenetic relationships between *A. platys* strains using several genes. Furthermore, the same holds true for phylogenetic sequence analysis comparing *A. platys* gene sequences with sequences from the Caribbean and other regions of the world. One study was recently carried out in dogs from the French Guyana region of the Caribbean and focused on detecting *A. platys* from dogs using conventional and quantitative PCR (Dahmani et al., 2015b). This, like other studies, did not perform any phylogenetic analyses, although a few 23S rRNA and 16S rRNA gene sequences are deposited in the NCBI database as unpublished work.

The current study was focused on assessing the infection status in dogs with no apparent clinical signs for *A. platys* and *E. canis* from the Caribbean island of Grenada and then mapping sequence similarities of *A. platys* isolates with other geographic locations around the world. Phylogenetic analyses were performed for *A. platys* sequences targeting 16S rRNA, 23S rRNA, *gltA* (citrate synthase), and *groEL* (Heat-shock protein) genes. Genetic diversity was compared between the newly reported sequences from this study, and other sequences of *A. platys* reported previously.

2. Materials and methods

2.1. Samples and genomic DNA extraction

A total of 155 blood samples drawn from the cephalic veins of apparently healthy dogs into EDTA tubes were collected from four parishes (St. Andrew, St. David, St. George, St. Mark) in Grenada during the years of 2017 and 2018. The samples were collected at the Junior Surgery laboratory and the St. George's University Small Animal Clinic. Genomic DNA was extracted from 100 μL volume of blood using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and eluted in a 100 μL volume of elution buffer, then stored at −20 °C.

2.2. PCR amplification of A. platys 16S rRNA, 23S rRNA, gltA and groEL genes

Firstly, dog genomic DNA samples were screened for the presence of any *Ehrlichia* and *Anaplasma* species using a generic primer pair (EHR16S forward and reverse) (Inokuma et al., 2000; Parola et al., 2000) (Table 1). A negative control PCR with no template DNA added was included each time a PCR assay was set up. Similarly, a known positive control reaction was set up, which contained *A. platys* genomic DNA as the template. PCRs were performed using a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, California, USA). Briefly, the first PCR was carried out in a 25 μl reaction mixture containing Platinum Taq DNA polymerase (Life Technologies, Carlsbad, CA, USA), 1X PCR Buffer (Life Technologies, Carlsbad, CA, USA), 100 μM dNTPs, 0.25 μM of each primer and 2 μl of DNA template (approximately 100 ng). PCR cycling conditions were as follows; initial denaturation at 94 °C for 3 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s, and then one cycle of extension at 72 °C for 2 min. The products were then stored at 4 °C.

The second PCR to detect *A. platys* or *E. canis* was carried out similarly as previously stated, with the only exception being gene/target specific primers. PCR thermal cycling conditions were done according to published protocols (Table 1). As *A. platys* 23S rRNA gene sequences were not available in the GenBank database at the time of our investigation, we compared 23S rRNA gene sequences representing *Anaplasma marginale* and *Anaplasma phagocytophilum* by sequence alignment to design primers from regions conserved in both species. Primers from the *Anaplasma* genus-specific regions were then used to amplify *A. platys* 23S rRNA gene segments. Sequences for oligonucleotide primers used to perform the above outlined PCRs and their predicted annealing temperatures as well as PCR product sizes are presented in Table 1. Products from the first and second PCR were resolved on a 1.5 % agarose gel to identify predicted amplicons. All PCR amplicons were purified using the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) and quantified using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Thereafter, both strands of the amplicons were sequenced by the Sanger sequencing method at MCLAB sequencing laboratory (San Francisco, CA, USA). Samples that tested positive for *Anaplasma* species by PCR were subsequently analysed for 16S rRNA, citrate synthase (*gltA*) and heat shock protein (*groEL)* gene sequences.

2.3. Sequence analysis and phylogenetic tree construction

Nucleotide sequences for the above-mentioned gene targets were edited, assembled and trimmed using Geneious Prime v2020.0.4 (Biomatters Ltd., Auckland, New Zealand,) and subsequently compared with existing sequences in the GenBank database using the BLAST algorithm (Altschul et al., 1990) on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences with the highest percentage identities with *A. platys* sequences on GenBank were aligned using the MUSCLE algorithm in MEGA X v10.1.7 (Kumar et al., 2018). Pairwise distances were computed in MEGA X for the *A. platys* 16S, 23S, *gltA* and *groEL* nucleotide sequences and also compared with *A. marginale*, *A. phagocytophilum*, *Ehrlichia ruminantium*, *E. canis* and *Ehrlichia chaffeensis*. Additionally, variance estimation for pairwise analysis for all four gene sequences was carried out by the bootstrap method with 1000 replicates and uniform evolutionary rates among sites. The 16S rDNA, 23S rDNA,

gltA and *groEL* nucleotide sequences were further model tested for Maximum Likelihood (ML), Neighbour Joining (NJ) and Unweighted Pair Group Method with Arithmetic mean (UPGMA) phylogeny methods in MEGA X. The Kimura 2-parameter model (K-2) (Kimura, 1980) with uniform evolutionary rates among sites and the K-2 model with discrete Gamma distribution among sites (K-2 + G) were found to be the best models for ML phylogeny for the 16S rDNA and 23S rDNA sequences, respectively. For both 16S and 23S sequences, NJ and UPGMA were performed using the K-2 model with uniform evolutionary rates as a basis. For *gltA* and *groEL* sequences, the T-92 parameter (Tamura 3-parameter) model (Tamura, 1992) with an evolutionary variable rate (T-92 + I) was determined to be the most appropriate for these data sets for ML, while the T-92 model with uniform evolutionary rates was best for NJ and UPGMA analysis. All phylogenetic analysis was implemented using the bootstrap method with 1000 replicates per tree. *In toto*, for each of the phylogenetic tree building methods (ML, NJ and UPGMA) the dataset with each of the four genes were tested in MEGA X to ascertain the model that was most suitable in generating the most reliable phylogenetic tree.

Homologous sequences from *E. ruminantium* (strain Welgevonden) were used as an outgroup for *A. platys* 16S rDNA, 23S rDNA, *gltA* and *groEL* (Accession numbers: NR 07413.2, NR 077000.1, NC005295.2 and CR767821.1 respectively) for ML and NJ analysis with rooted trees generated. We also used *A. platys* 16S rDNA, 23S rDNA, *gltA* and *groEL* sequences from the recently published S3 strain of *A. platys* (Accession number CP04639) from St. Kitts, West Indies (Llanes and Rajeev, 2020), which represents the first *A. platys* genome to be sequenced. Additionally, homologous *A. marginale* (Accession no.: CP001079.1 and CP001079), *A. phagocytophilum* (Accession no.: CP000235.1 and APHH0100001.1), *E. canis* (Accession no.: CP000107.1, NR 076375 and CP025749), and *E. chaffeensis* (Accession no.: CP000236.1 and NR 076400) whole genome sequences were included in the phylogenetic analysis to increase tree robustness.

3. Results

3.1. Assessment of canine blood samples randomly collected from Grenada for Ehrlichia and Anaplasma species pathogen infections

One hundred and fifty-five canine blood samples randomly collected from different locations in St. Georges, Grenada, West Indies were assessed by genera-specific PCR targeting *Ehrlichia* and *Anaplasma* species 16S rRNA gene segment. Sixty-six samples (42.6 %) tested positive for the predicted amplicons of 345 bp, which were then subjected to DNA sequence analysis. Twenty-nine of the 66 positive samples tested positive for *A. platys*, while 26 of the remaining samples were identified as *E. canis* sequences. Due to poor sequence quality, we were unable to obtain the identities of the remaining 11 samples. The overall infection prevalence rate of 18.7 % (29/155) for *A. platys* and 16.8 % (26/155) for *E. canis* (Table 2). The *A. platys*-positive samples were further evaluated by subjecting them to sequence analysis targeting 23S rRNA, *gltA* and *groEL* genes. *Anaplasma platys* 16S rDNA sequences showed >99 % sequence identity with previously reported sequences representing diverse geographic regions (Table 3). Samples from the St. George parish of Grenada had the highest overall *A. playts* infection prevalence; 30.8 % (12/39), which is nearly double the

overall infection prevalence observed for the samples representing other parishes of the island (Table 2). Although St. David parish samples had a higher prevalence (50 %), the number of samples from this location were low (Table 2). Samples from St. Andrews, St. Mark and of unknown origin in Grenada had comparable infection prevalences ranging from 11 to 17%.

3.2. Phylogenetic analysis

A total of 28, 12, 21 and 18 sequences were used in the generation of the final phylogenetic trees for 16S, 23S, *gltA* and *groEL* sequences, respectively. A robust ML phylogenetic trees (and NJ and UPGMA trees also) for *A. platys* and other *Anaplasma* species sequences were generated as supported by the high bootstrap values of 99–100 % on the main internal tree nodes for the 16S rRNA, 23S rRNA, *gltA* and *groEL* gene sequences. The data correspond well with pairwise analysis data, which show low genetic distance or high similarity between *A. platys* sequences. Pairwise comparison of *A. platys* sequences with other closely related *Ehrlichia* and *Anaplasma* species from GenBank are presented as supplementary data set (Supplementary Tables 1–4 and Supplementary Data 1–4). The pairwise comparisons showed average nucleotide substitutions of: 0.000–0.059 (*16S*), 0.051–0.111 (23S), 0.000–0.977 (*gltA*) and 0.000–0.391 (*groEL*). As a general observation, *Ehrlichia* and *Anaplasma* species sequences clustered into distinct branches according to species and geographic origin for 16S rDNA and *gltA* phylogenetic trees (Figs. 1 and 3, respectively), whereas the 23S rDNA and *groEL* gene sequences (Figs. 2 and 4, respectively) grouped together into distinct clusters by species, regardless of the region of the world they originate from. All representative *A. platys* sequences generated in this study were submitted to GenBank under the accession numbers MW450800–MW450814.

4. Discussion

The current study demonstrates the high prevalence of circulating *A. platys* and *E. canis* organisms in dogs and validates prior studies regarding the pathogens' presence identified mostly by serology from the Caribbean island of Grenada (Wilkerson et al., 2017; Lanza-Perea et al., 2014; Yabsley et al., 2008). Further, the study demonstrates that some dogs on the island have active infections while exhibiting no apparent clinical signs. Similar studies reporting PCR positives in asymptomatic dogs have also been conducted in the region, in St. Kitts (Lara et al., 2020; Kelly et al., 2013; Loftis et al., 2013) and Nicaragua (Springer et al., 2018; Wei et al., 2015). These studies further highlight the occurrence and extent of this phenomenon as well as the continued need to monitor such asymptomatic dogs in Grenada and in other parts of the world where the infections may be prevalent. The study also builds on the previous data through targeted sequence analysis of four different *A. platys* genes, which further confirms the pathogen's existence by phylogenetic analysis. The data also suggest the importance of rickettsial infections in mostly free-roaming dogs, with *A. platys* and *E. canis* serving as potential sources for zoonotic infections for people. No observable and obvious clinical manifestations were apparent in dogs, and these tested positive for the circulating *A. platys* and/or *E. canis* organisms, which is similar to a previous study in Grenada (Lanza-Perea et al., 2014). The current study tested for the presence of organisms detectable by molecular analysis and differs from the previous study, which reported

seropositives in apparently healthy dogs. Thus, the present study is the first in Grenada documenting detailed molecular evidence for actively circulating *A. platys* and *E. canis* organisms in dogs with no observable clinical signs.

Infected animals pose a potential public health problem as they could be asymptomatic, thus posing a challenge for diagnosis, since they may be erroneously missed and not be part of routine investigations (Harrus et al., 1997b; Harvey et al., 1978). Previous studies demonstrate that *A. platys* infects a range of host species. Besides dogs, the pathogen is reported to infect cats, cattle and people (Dahmani et al., 2015a; Arraga-Alvarado et al., 2014; Qurollo et al., 2014; Maggi et al., 2013; Lima et al., 2010). Human infections with *E. canis* have also been recorded in Venezuela and the USA (Perez et al., 2006, 1996; Taylor et al., 1988; Fishbein et al., 1987; Maeda et al., 1987)

Dogs with high amounts of actively circulating rickettsial pathogens may serve as important reservoir hosts, thus enabling their consistent spread among dogs, other vertebrates and ticks. The risk of humans acquiring *A. platys* and *E. canis* is, therefore, enhanced because dogs are the closest companion animals, independent of their status as free-roaming dogs owned by people in places such as in the Caribbean. Dogs may thus represent an environmental and public health sentinel species to track potential human infections (Reif, 2011; Cleaveland et al., 2006).

The current study location was in tropical climatic conditions where infections with rickettsial pathogens are known to occur in many climatically similar geographic regions of the world in India, Argentina, Brazil and the French Guyana (Manoj et al., 2020; Soares et al., 2017; Cicuttin et al., 2015; Dahmani et al., 2015b). *Anaplasma platys* and *E. canis* infections are expected in the Caribbean as recent studies also demonstrated their existence in Grenadian dogs (Wilkerson et al., 2017; Lanza-Perea et al., 2014; Yabsley et al., 2008). Our study revealed an infection prevalence of 18.7 % for *A. platys* and this was comparable to data from another earlier study from Grenada (Yabsley et al., 2008) and French Guyana (Dahmani et al., 2015b). The aforementioned studies reported infection prevalences of 19.2 % (14/73) for *A. platys* by PCR targeting a segment of the 16S rRNA gene (Yabsley et al., 2008) and similarly, 15.4 % (10/65) for the 23S rRNA gene segment, also by PCR analysis (Dahmani et al., 2015b). In our current study, we performed a detailed molecular analysis involving PCRs targeting four different gene segments followed by DNA sequence analysis and sequence comparisons to diverse geographic isolates of *A. platys*, using 155 dog blood samples. Prior PCR-based evidence is also documented for *A. platys* infection in dogs from various geographic regions of the world, including Brazil (approximately 16 %) (Soares et al., 2017), Mexico (approximately 10 %) (Almazán et al., 2016), USA (4.5–8.3 %) (Diniz et al., 2010), Portugal (three out of four clinically sick animals tested positive) (Cardoso et al., 2010), Malaysia (about 3%) (Low et al., 2018), India (45/230; about 20 %) (Manoj et al., 2020). Infection prevalence in Brazil and India appear to be similar to the current data. Co-infection of dogs with both *E. canis* and *A. platys* is not uncommon. In the current study, our data showed a co-infection rate of 4.5 % for *A. platys* and *E. canis*. Three PCR-based canine studies in this region of the world also reveal *E. canis* and *A. platys* co-infection rates of 19 % in St. Kitts (Lara et al., 2020), 4.7 % in Nicaragua (Springer et al., 2018) and 5% in Costa Rica (Wei et al., 2015). While the St. Kitts study shows a much higher co-infection rate, the

Nicaragua and Costa Rica studies show very similar co-infection rates with our study. In a previous study in Grenada using ELISA and IFAT, we reported co-seropositivity in dogs for *A. platys* and *E. canis* to be 13 % and 9%, respectively (Wilkerson et al., 2017).

In the recent aforementioned study in St. Kitts (Lara et al., 2020), an antibody-based bead assay detected a co-seropositivity rate of 22 % in dogs. These two antibody based test results are expected as immunological assays are more sensitive and also known to yield more positives due to higher cross-reactions with related rickettsia compared to PCR-based assays (Biggs et al., 2016). Furthermore, the accuracy of these serological assays can vary widely between testing laboratories primarily due to the lack of standardized antigenic targets, cross-reactivity, and subjective establishment of positivity thresholds (Biggs et al., 2016). The difference in results in this and other aforementioned studies may also be explained by variations in experimental factors such as sample numbers, types of assays used to assess infection status and seasonal factors supporting different tick abundances.

The brown dog tick is regarded as the most widespread tick species in the world (Dantas-Torres, 2010) and is often associated with dogs (Latrofa et al., 2014), including Grenada (Yabsley et al., 2008). This tick is the likely vector for *A. platys* transmission and is also the known vector for *E. canis* and other important tick-borne pathogens impacting canine and human health (Inokuma et al., 2000; Kordick et al., 1999). In this study, we observed the presence of this tick on nearly all the dogs sampled. Warm and humid tropical climatic conditions that exist in Grenada are favourable for survival and spread of the brown dog tick. This is supported by the previous study reporting the prevalence of *R. sanguineus* s.l. from nearby Caribbean islands; St. Kitts (Loftis et al., 2013) and Haiti (Starkey et al., 2016). Likewise, the tick's prevalence is reported from a southern part of India where comparable climatic conditions exist (Manoj et al., 2020). This tick prefers to feed on one host, the domesticated dog, and it is ubiquitous in and around homes. Nonetheless, it is adaptable to parasitizing people in the event that it is unable to find a canine host or when humans are encountered accidentally (Dantas-Torres, 2010; Dantas-Torres et al., 2006). Indeed, several studies documented parasitism of this tick on humans, mostly from the western hemisphere; in Brazil (Dantas-Torres et al., 2006), Uruguay (Venzal et al., 2003), Argentina (Guglielmone et al., 1991), USA (Carpenter et al., 1990) and Italy (Manfredi et al., 1999). Studies also reported fatal outbreaks of Rocky Mountain spotted fever resulting from *R. rickettsii*-infected *R. sanguineus* s.l. in the Americas, i.e. in Arizona in the USA (Eremeeva, 2012; Openshaw et al., 2010; Demma et al., 2006, 2005; Nicholson et al., 2006), northern Mexico (Álvarez-Hernández et al., 2017; Eremeeva et al., 2011; Tinoco-Gracia et al., 2009) and Brazil (Cunha et al., 2009; Moraes-Filho et al., 2009). Importantly, recent evidence suggests that this tick vector is likely more significant as a human ectoparasite and also in transmitting pathogens than previously regarded (Paddock et al., 2016; Eremeeva, 2012). The presence of this tick is, therefore, a significant threat to both canine and human health, and thus requires monitoring in and around human dwellings as well as on their companion animals, particularly on dogs (Dantas-Torres et al., 2013).

The phylogenetic data presented in this study for the four gene targets; 16S and 23S rRNA, *gltA* and *groEL* genes of *A. platys*, are in agreement with the taxonomic separation of members of the family *Anaplasmataceae* into the *Ehrlichia* and *Anaplasma* genera, as well

as other genera (*Neorickettsia* and *Wolbachia*) (Dumler et al., 2001). Our phylogenetic analysis demonstrates that *A. platys* sequences cluster tightly into an *A. platys* subclade, followed by *A. marginale* along with *A. phagocytophilum* clustering into their own subclades, in accordance with previously identified *Anaplasma* species similarities (Llanes and Rajeev, 2020; Dumler et al., 2001; Inokuma et al., 2001). Additionally, the *A. platys* clade containing nucleotide sequences of both 16S rRNA and *gltA* genes showed similar topology for these phylogenetic trees as they branched out into two distinct sub-groups/clades, A and B (Figs. 1 and 3), in which sequences grouped together according to geographic origin of the samples. The SK-072 and S3 strains of *A. platys* from St. Kitts, which sit in different clades seem to be indicative of sufficient genetic divergence for these to be placed together with strains from different geographical locations of the world, according to the 16S rRNA gene phylogenetic tree. Despite the accuracy of these sequences, however, this result would have been more reliable if 23S rRNA, *gltA* and *groEL* gene sequences from the SK-072 were also available for an assessment of where they sat relative to sequences from other geographic locations, in the respective phylogenetic trees. Subclade A, which we designated the 'Americas group', contained the Grenada sequences reported in this study, as well as other Carribean origin sequences reported earlier from Trinidad and St. Kitts (16S rRNA gene sequence from the St. Kitts SK-072 strain *A. platys* with accession number, JX112781). Sequences in this clade also included sequences from these regions of the world as well those documented from Panama, Peru, Colombia, Cuba and USA (Central, South and North America). Subclade B contained sequences from Africa, Europe and Asia (with the exception of one 16S sequence from the St. Kitts strain of *A. platys*; accession number CP046391) and therefore, it was designated the 'Africa/Eurasia group', with some of the geographically representative sequences being from South Africa, Italy and Japan, respectively. This clustering of *A. platys* 16S rRNA and *gltA* gene sequences into subclades A and B suggests a high phylogeographic sequence similarity for these gene sequences, which may point to common origins/ancestry of the two major representative strains documented from this region of the world. This separation into subclades A and B in the 16S rRNA and *gltA* phylogenetic trees may also imply that two major genetic strains of *A. platys* with minor variations are circulating in the world, although more data from other regions of the world are needed to reach a more informative conclusion. Genome sequencing or mutli-locus sequence typing of several global strains may also be conducted to shed light on the variation in these and other world strains of *A. platys*. Furthermore, high sequence similarities observed in strains from different geographical regions in the aforementioned Americas and Africa/Eurasia group clades, may also indicate recent movements of the *A. platys* pathogen via infected vertebrate hosts and/or tick vectors.

The *A. platys* 23S rRNA and *groEL* gene sequences each grouped into one main *A. platys* subclade regardless of geographic origin, which was not expected. This may be explained by the fact that the sequence variations in the gene loci we used for the phylogenetic tree construction may have had inadequate discriminatory power to separate the *A. platys* strains. In particular, for the 23S rRNA gene, only few sequences were available in the GenBank database. A potential solution, which may be explored in future studies, is to generate longer PCR amplicons to get more informative sequence data, which may prove to have higher discriminatory power to separate the strains based on 23S rRNA and *groEL* genes.

5. Conclusion

This work corroborates findings from previous studies as it provides more detailed molecular evidence of *A. platys* and *E. canis* infections in apparently healthy dogs from the St. George's region of Grenada, and also sheds light on the phylogenetic relationships between the Caribbean and other global strains of *A. platys*, while highlighting the importance and continued utility of molecular methods in performing such studies. Additionally, this study reiterates the need to raise awareness about the presence of *A. platys* and *E. canis*, which are among important rickettsial zoonotic pathogens in this area. We anticipate that the information gleaned from this study will build on current data and provide further information on the presence and genetic diversity of *A. platys* in dogs in the Caribbean, including in Grenada and the regions of the world where these pathogens are more prevalent.

Supplementary Material

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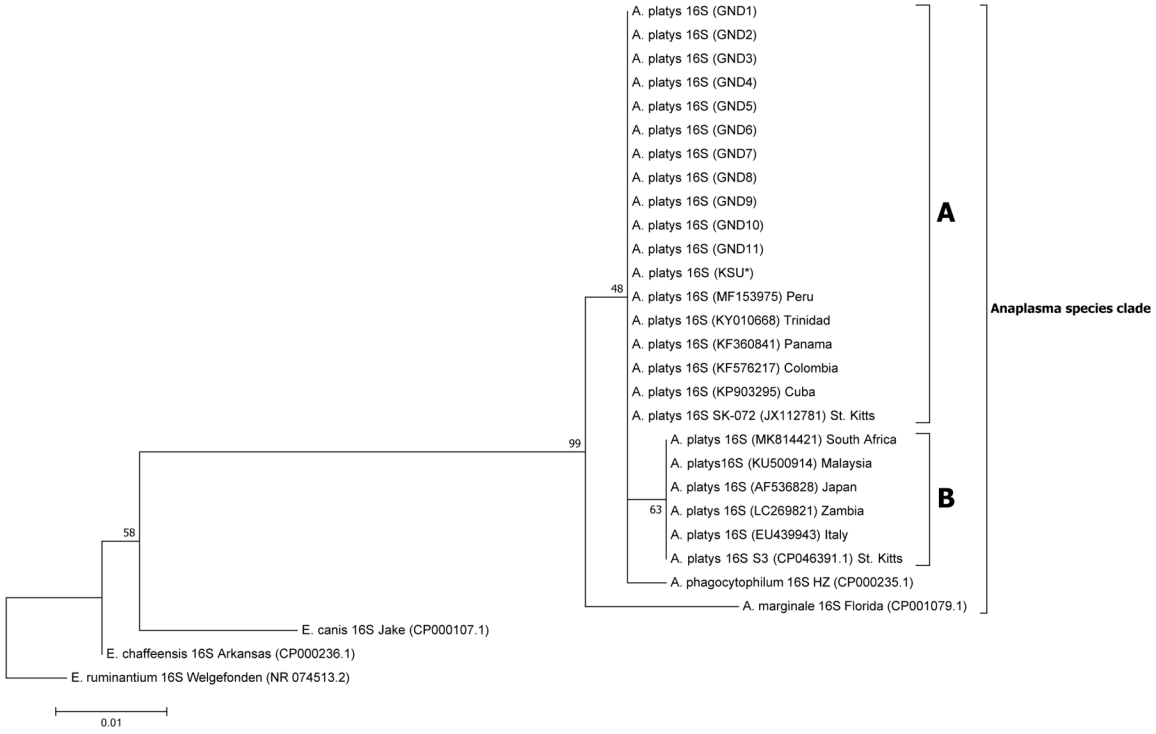

Fig. 1.

Maximum likelihood (ML) phylogenetic analysis of *A. platys* 16S rRNA gene sequences. Phylogenetic relationships between *A. platys* and homologous 16S sequences from *A. marginale*, *A. phagocytophilum*, *E. canis*, *E. chaffeensis* and *E. ruminantium* were established. Bootstrap values are shown by numbers at each internal node and these represent the percentage of 1000 replicates for which the same branching pattern was obtained. There were a total of 1456 positions in the dataset. Both NJ and UPGMA analyses for 16S sequences generated trees with similar topology with those derived from ML analysis (Supplementary Fig. 1a and b). A total of 29 sequences were used in the final analysis and the scale-bar represents a 1% nucleotide sequence divergence. Pairwise identity results of these data are given in Supplementary Table 1 and Data 1. (KSU*) = sample obtained from a dog tested positive in Florida, USA. GND = Grenada samples.

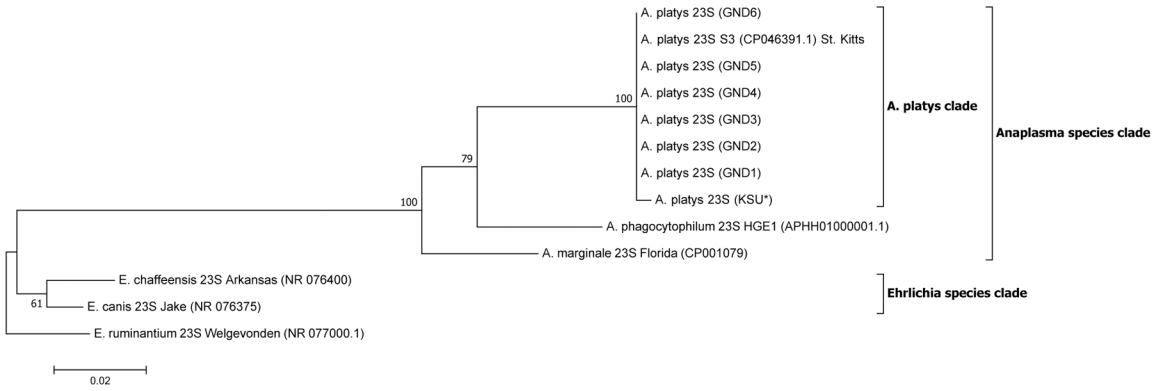

Fig. 2.

Maximum likelihood (ML) phylogenetic analysis of *A. platys* 23S rRNA gene sequences. Phylogenetic relationships between *A. platys* and homologous 23S sequences from *A. marginale*, *A. phagocytophilum*, *E. canis*, *E. chaffeensis* and *E. ruminantium* were established. Bootstrap values are shown by numbers at each internal node and represent the percentage of 1000 replicates for which the same branching pattern was obtained. There were a total of 2794 positions in the dataset. Both NJ and UPGMA analyses for 23S sequences generated trees with similar topology with those derived from ML analysis (Supplementary Fig. 2a and b). A total of 13 nucleotide sequences were used in the final analysis and the scale-bar represents a 2% nucleotide sequence divergence. Pairwise identity results of these data are given in Supplementary Table 2 and Data 2. (KSU*) = sample obtained from a dog tested positive in Florida, USA. GND = Grenada samples.

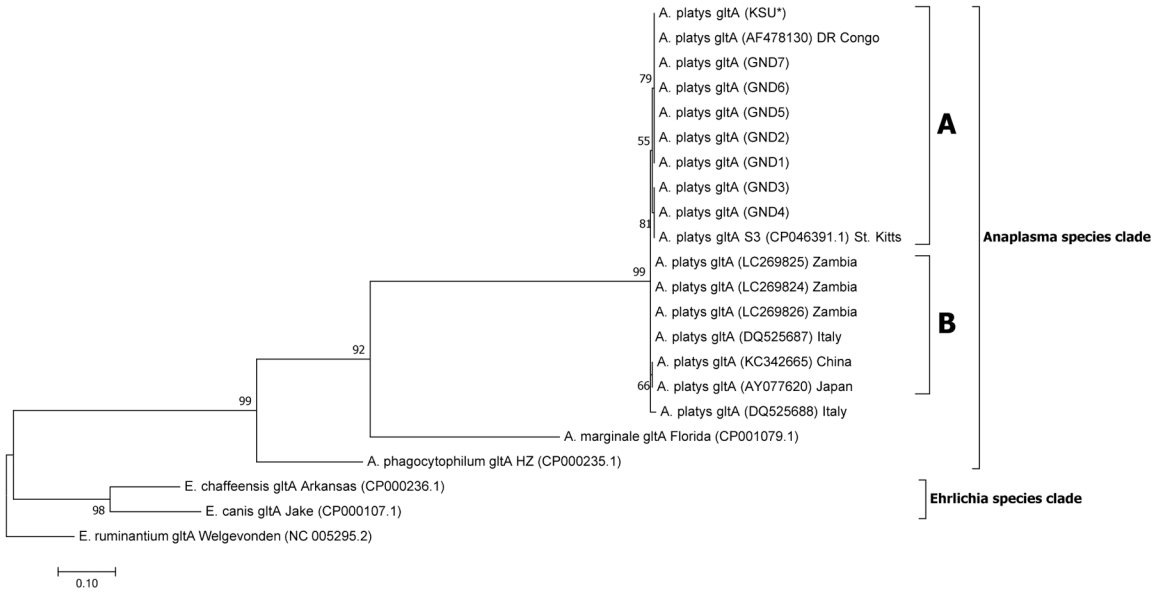

Fig. 3.

Phylogenetic analysis of *A. platys gltA* gene sequences. Phylogenetic relationships between *A. platys* and homologous *gltA* sequences from *A. marginale*, *A. phagocytophilum*, *E. canis*, *E. chaffeensis* and *E. ruminantium* were established. Bootstrap values are shown by numbers at each internal node and represent the percentage of 1000 replicates for which the same branching pattern was obtained. There were a total of 1287 positions in the dataset. The analysis was performed as in Fig. 2. For additional details, please refer to Supplementary Fig. 3a and b. A total of 21 nucleotide sequences were used in the final analysis and the scale-bar represents a 10 % nucleotide sequence divergence. Pairwise identity results are included in Supplementary Table 3 and Data 3.

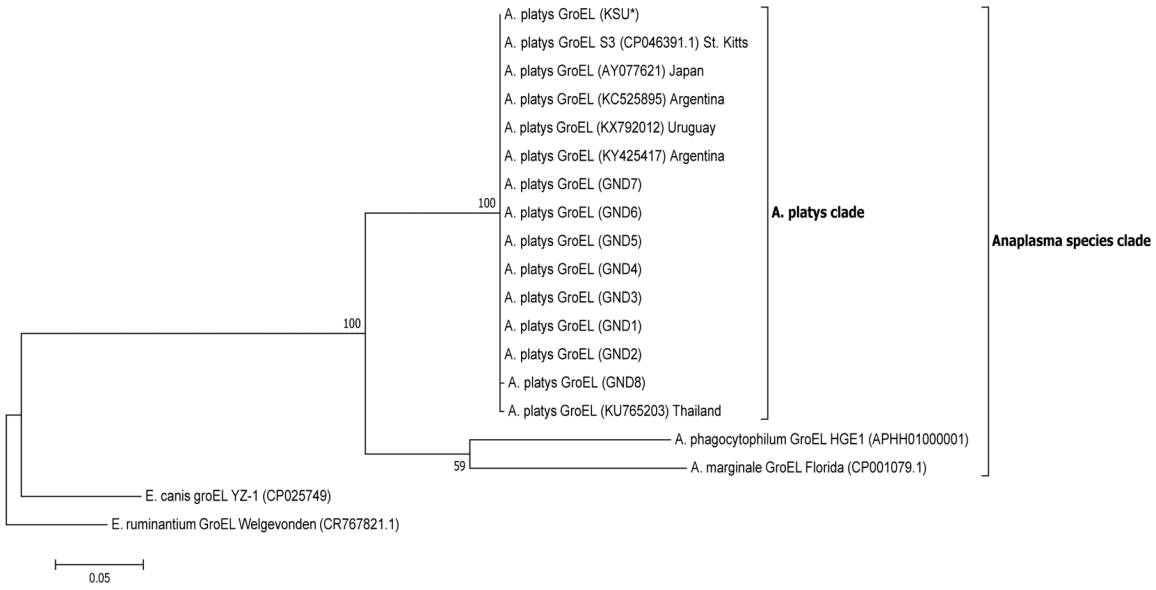

Fig. 4.

Phylogenetic analysis of *A. platys groEL* gene sequences. Phylogenetic relationships between *A. platys* and homologous *groEL* sequences from *A. marginale, A. phagocytophilum*, *E. canis*, *E. chaffeensis* and *E. ruminantium* were established. Bootstrap values are shown by numbers at each internal node and represent the percentage of 1000 replicates for which the same branching pattern was obtained. There were a total of 1653 positions in the dataset. The analysis was performed as in the previous two figures. Additional details are provided in Supplementary Fig. 4a and b. A total of 19 nucleotide sequences were used in the final analysis and the scale-bar represents a 5% nucleotide sequence divergence. Pairwise identity results are included in Supplementary Table 4 and Data 4.

Table 1

PCR primers used for amplifying various gene segments *of Ehrlichia* and *Anaplasma* species in this study.

Name	Sequence	Annealing Temperature	Size (bp)	Reference(s)
EHR16SF	GGTACCYACAGAAGAAGTCC	55 °C	345	Inokuma et al., 2000; Parola et al., 2000
EHR16SR	TAGCACTCATCGTTTACAGC			
Aplatys.16S-F	TTTGTCGTAGCTTGCTAT	50 °C	349	Matei et al., 2016
Aplatys.16S-R	CTTCTGTGGGTACCGTC			
Aplatys-23sF2	TCGATGGGAATCAGGTTAATATTCCTG	55 °C	800	This study
Aplatys-23sR2	TTGTACTATAAAAGCTGATTTCCG			
Aplatys-groEF	AAGGCGAAAGAAGCAGTCTTA	54 °C	724	Inokuma et al., 2002
Aplatys-groER	CATAGTCTGAAGTGGAGGAC			
Aplatys-gltAF	GACCTACGATCCGGGATTCA	61 °C	580	Silva et al., 2016
Aplatys-gltAR	CCGCACGGTCGCTGTT			
E. canis F	CAATTATTTATAGCCTCTGGCTATAGGA	58 °C	475	Rufino et al., 2013
E. canis R	ATAGGGAAGATAATGACGGTACCTATA			

Table 2

Location and number data for canine blood samples used in this study.

Parish	**Total samples**	***Ehrlichia/ Anaplasma* spp. (%)**	***E. canis* (%)**	***A. platys* (%)**
St. Andrew	54	15 (27.8)	9 (16.6)	6 (11.1)
St. David	4	3 (75.0)	1 (25.0)	2 (50.0)
St. George	39	20 (51.3)	8 (20.5)	12 (30.8)
St. Mark	12	6 (50.0)	3 (25.0)	2 (16.7)
Unknown	46	22 (48.0)	5 (10.9)	7 (15.2)
Total	**155**	**66 (42.6)**	**26 (16.8)**	**29 (18.7)**

NB: *Ehrlichia* and *Anaplasma* species., *E. canis* and *A. platys* % values were calculated as a % of total number of samples.

Table 3

Selected 16S rRNA gene sequences from GenBank matching *A. platys* isolates from this study.

Accession numbers	Identity (%)	Country
MK506833	99.48	Cuba
MF153975	99.48	Peru
MK814421	99.22	South Africa
LC269821	99.22	Zambia
KU500912	99.22	Malaysia
JX118826	99.22	Brazil
AF286699	99.22	Thailand
KX818218	98.92	India
AF303467	99.48	France
AF536828	99.48	Japan
M82801	99.48	USA
KY010669	99.48	Trinidad