Heliyon 6 (2020) e04542

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Application of 16S rRNA next generation sequencing in ticks in Greece

Anna Papa [a,*], Katerina Tsioka [a], Maria-Antonia Daskou [a], Fani Minti [a], Elpida Papadopoulou [a], Ageliki Melidou [a], Nektarios Giadinis [b]

[a] Department of Microbiology, Medical School, Aristotle University of Thessaloniki, Greece
[b] Clinic of Farm Animals, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, Greece

ARTICLE INFO

Keywords:
Microbiology
Epidemiology
Infectious disease
Laboratory medicine
Medical microbiology
Microbiology epidemiology
Ticks
Bacteria
16S rRNA
Next generation sequencing
Bacteriome
Greece

ABSTRACT

Tick-borne bacteria pose a significant threat to human and veterinary public health. Greece is a Mediterranean country with rich tick fauna and the most commonly detected tick-borne bacterial pathogens are members of the *Rickettsia* and *Anaplasma* species. The variable V2–V4 and V6–V9 regions of 16S rRNA gene of seven ticks belonging to four genera representative in Greece (*Ixodes, Rhipicephalus, Dermacentor, Haemophyssalis*) were analysed using multiple primer pairs by next generation sequencing (NGS). Nine bacterial phyla corresponding to 95 families, 116 genera and 172 species were identified. Proteobacteria was the predominant phylum in five of the seven ticks, followed by Actinobacteria, which predominated in two ticks. The tick-borne bacteria included *Rickettsia* and *Anaplasma* species, while "*Candidatus* Midichloria mitochondrii" were detected in high abundance in *I. ricinus* ticks and less in *Rhipicephalus bursa*; *Coxiella*-like endosymbionts were detected in *Rh. sanguineus*, *H. parva*, and less in *Rh. bursa* ticks. Co-infections with *Rickettsia* and *Anaplasma* were also observed. 16S rRNA NGS is a powerful tool to investigate the tick bacteriome and can improve the strategies for prevention and control of tick-borne diseases.

1. Introduction

Ticks (class Arachnida, subclass Acari, order Ixodida) are hematophagous arthropods that are second to mosquitoes as vectors of human pathogens and the most important vectors of pathogens in domestic and wild animals (Parola and Raoult, 2001). Among tick-transmitted bacterial pathogens are multiple *Rickettsia, Anaplasma, Ehrlichia*, *Bartonella, Borrelia* and *Francicella* species that are recognized as significant threats to human and veterinary public health (Wikel, 2018). Besides pathogenic bacteria, several bacterial endosymbionts are detected in ticks which interact in a constant and complex way (Wernegreen, 2012).

Until recently, the detection of bacteria in ticks was achieved mainly by application of individual or multiplex molecular methods. Since 2011, when next generation sequencing (NGS) was applied for the study of tick microbiome (Andreotti et al., 2011), the number of metagenomic studies in ticks is increasing. Over the past few years, the availability of NGS technologies enabled the rapid, high-throughput parallel sequencing of diverse microbial communities (Couper and Swei, 2018). The sequencing of 16S rRNA NGS technique targets the highly conserved 16S rRNA gene of bacteria and archaea. Specifically, the hypervariable regions V1–V9 differ between species; therefore, their sequencing enables the identification of microorganisms and their classification at the family, genus and species level (Greay et al., 2018). Since the bacteriome includes not only pathogenic bacteria, but also a great variety of symbionts, it is apparent that NGS technology enables the broader elucidation of tick-microbiome-host interactions which play a critical role in tick survival, vector competence, pathogen transmission and host susceptibility to tick-borne infections (de la Fuente et al., 2017).

A study on species diversity and spatial distribution of ixodid ticks collected from small ruminants in Greece detected two species of *Rhipicephalus*, two of *Ixodes*, five of *Hyalomma*, three of *Haemaphysalis*, and one of *Dermacentor*, with *Rhipicephalus sanguineus* s.l. and *Rh. bursa* accounting for 64.8 % and 25.9 % of ticks, respectively (Chaligiannis et al., 2016). The most commonly reported tick-borne bacterial pathogens in Greece are members of the *Rickettsia* and *Anaplasma* species. In a recent study in Greece, several bacterial pathogens (*Rickettsia monacensis, R. massilae, Anaplasma phagocytophilum* and *A. platys*), as well as endosymbiots [*Coxiella*-like endosymbionts (CLEs) and "*Candidatus* Midichloria mitochondrii" (CMM) were detected in ticks (Papa et al., 2017)].

The aim of the present study was to apply 16s rRNA NGS approach to explore tick microbiome of hard ticks by sequencing of the hypervariable regions of 16S rRNA gene using NGS technique and to compare the

* Corresponding author.
E-mail address: annap@med.auth.gr (A. Papa).

https://doi.org/10.1016/j.heliyon.2020.e04542
Received 4 March 2020; Received in revised form 10 June 2020; Accepted 21 July 2020

results with those of a previous study in which conventional molecular methods were applied.

2. Materials and methods

Stored (in -80 °C) DNA samples previously extracted from seven individual ticks (S1–S7) belonging to four genera (*Ixodes, Rhipicephalus, Dermacentor, Haemophyssalis*) were subjected to 16S rRNA NGS. All ticks had been collected from goats, except S4 which was collected from a sheep, in the frame of a study conducted during 2015–2016 (Papa et al., 2017). The selection of the material was based on inclusion of representative tick species in Greece. In three ticks at least one pathogen was previously detected (S1: *R. monacensis* and *Anaplasma platys*; S2: *Rickettsia monacensis*: S4: *R. massiliae* and *A. ovis*), while no pathogen was detected in the remaining four ticks (S3, S5-T7) (Table 1).

The hypervariable regions of 16S rRNA gene were amplified using the Ion 16S rRNA Metagenomics Kit (Life Technologies Corporation, Carlsbad, CA, USA). For each sample two separate multiplex PCR assays were applied, one that targeted the hypervariable regions V2, V4 and V8, and one that targeted the regions V3, V6/7 and V9. Following library preparation, the samples were loaded in an Ion 316 Chip v2 and sequencing was performed on an Ion Personal Genome Machine (PGM) system using the Ion PGM Hi-Q (400) sequencing kit (Life Technologies Corporation, Carlsbad, CA, USA). The data were analyzed with Ion Reporter software and Krona diagrams were generated. Furthermore, the fasta files were analyzed using the BLAST tool (https://blast.ncbi.nlm.nih.gov/).

Statistical analysis of data was performed using the open source R programming language 3.3.1v. The vegan 2.4.2v (Oksanen et al., 2016) and phyloseq 1.19.1v R packages were imported for data handling, while for data visualization the ggplot2 package was applied (McMurdie and Holmes, 2013; Wickham, 2016). The relative abundance of taxa was explored with bar and heat map plots.

3. Results

A total of 2,433,414 raw reads were generated. The study was submitted to European Nucleotide Archive (ENA) and the accession numbers together with the quality control (QC) data per sample are shown in Table 2.

Nine bacterial phyla corresponding to 95 families, 116 genera and 172 species were identified. The relative abundance of individual phyla per tick is displayed in Figure 1. Proteobacteria was the predominant phylum in five of the seven ticks (S1, S2, S4, S5 and S7), followed by Actinobacteria, which predominated in two ticks (S3 and S6). Specifically, Proteobacteria accounted for 75.13% with 29 families, and Actinobacteria accounted for 14.96% with 22 families. They were followed by Firmicutes (4.81%, 25 families) and Bacteroidetes (3.76%, 10 families), while the remaining five phyla (Cyanobacteria, Tenericutes, Fusobacteria, Spirochaetes and Chloroflexi) contributed lower than 2.0% to the total abundance. Among Proteobacteria, Alphaproteobacteria predominated in *I. ricinus* and one *Rh. bursa* (S4), while Gammaproteobacteria predominated in the *Rh. sanguineus* tick (S5).

At family level, "*Candidatus* Midichloriaceae" and *Rickettsiaceae* (both belonging to Proteobacteria) predominated with 44.22% and 15.86% of the total reads, and were detected in five and three ticks, respectively (Table 3A). Specifically, bacteria of the "*Candidatus* Midichloriaceae" family were mainly detected in *I. ricinus* ticks, while bacteria of the *Rickettsiaceae* family were detected in *I. ricinus* ticks (S1 and S2), and in one *Rh. bursa* tick (S4). These results are in accordance with the previous study (performed using individual conventional PCRs), in which *R. monacensis* was detected in the *I. ricinus* ticks and *R. massiliae* in one *Rh. bursa* tick (Table 1). Members of two other families, *Corynebacteriaceae* (mainly *Corynebacterium* spp.) and *Coxiellaceae* (mainly *Coxiella* spp.) were detected in all seven ticks in various abundance levels: bacteria of the *Corynebacteriaceae* family were mainly detected in *Rh. bursa* (S3) and *D. marginatus* ticks (S6), while bacteria of *Coxiellaceae* family were mainly detected in *Rh. sanguineus* (S5) and *H. parva* (S7), and in lower level in *Rh. bursa* ticks (S3 and S4). Sequences of *Anaplasmataceae* family were detected in *I. ricinus* ticks (S1 and S2), one *Rh. bursa* (S4) and in the *D. marginatus* tick (S6).

The abundance of the ten most prevalent genera per tick is shown Table 3B and in the heatmap (Figure 2), while the genera detected in each tick are shown in Table 4. Comparing the results taken by NGS with those from the previous study, all pathogenic bacteria were detected by both approaches, except the detection of *Anaplasma* spp. in one tick (S2), which was detected only by NGS (Table 1).

4. Discussion

To identify the bacteriome in representative tick species in Greece, 16S rRNA NGS was applied and the results were compared with those of a previous study in which ticks were tested for several pathogens using conventional PCRs. A variety of pathogenic and symbiotic bacteria were identified, and it was shown that the bacterial composition differed between species and even between ticks of the same species. Co-infections with more than one tick-borne pathogen were also observed. Since ticks may harbor a variety of microorganisms, including eukaryotes, bacteria and viruses, co-infection patterns have been described (Cross et al., 2018). As expected, these co-infections are most often indentified when NGS technologies are applied.

The tick-borne bacteria detected in the two female *I. ricinus* ticks of the present study (S1 and S2) belonged to the *Rickettsia* and *Anaplasma* species. Due to the conserved nature of the *Rickettsia* genus at the 16S rRNA gene (Roux and Raoult, 1995) resolution to species level was not provided in the NGS report. However, testing by Rickettsia-specific PCR and sequencing, *Rickettsia* was identified as *R. monacensis* (in both ticks) (Papa et al., 2017). *Anaplasma* sequences accounted for 1.24% of reads in S1, and BLAST analysis gave evidence that it was *A. platys,* which coincides with the results of the conventional PCR. In S2, the respective percentage was low (0.19%), explaining why *Anaplasma* sequences were not detected by PCR in this sample. BLAST analysis showed that the

Table 1. Tick-borne bacteria and endosymbionts detected in the ticks of the study. CMM: *Candidatus* Midichloria mitochondrii; CLE: *Coxiella*-like endosymbionts.

Sample	Tick species	Sex	Location	Date	Bacteria detected			
					Pathogens		Endosymbionts	
					PCR	16S rRNA NGS	PCR	16S rRNA NGS
S1	*I. ricinus*	F	Chalkidiki	Mar 2015	*R. monacensis* + *A. platys*	*Rickettsia* spp. + *A. platys*	CMM	CMM
S2	*I. ricinus*	F	Serres	Feb 2015	*R. monacensis*	*Rickettsia* spp. + *A. ovis*	CMM	CMM
S3	*Rh. bursa*	M	Thessaloniki	Jul 2015			CLE	CLE
S4	*Rh. bursa*	M	Thessaloniki	Jun 2015	*R. massiliae* + *A. ovis*	*Rickettsia* spp. + *A. ovis*	CMM + CLE	CMM + CLE
S5	*Rh. sanguineus*	F	Serres	Oct 2016			ND	CLE
S6	*D. marginatus*	F	Pella	Mar 2015	not tested	*A. ovis*		
S7	*H. parva*	F	Serres	Apr 2015			CLE	CLE

Table 2. Quality control data and ENA accession numbers of the samples of the study.

Sample	Bases	>=Q20 bases	Reads	Mean read length	Acc. number
S1	42,858,900	39,293,897	193,839	221	ERS4662531
S2	149,883,405	135,997,889	780,615	192	ERS4662535
S3	47,240,132	43,130,665	215,364	219	ERS4662534
S4	12,411,189	11,356,848	65,588	189	ERS4662533
S5	44,111,313	40,816,276	196,943	224	ERS4662532
S6	44,403,968	40,985,202	196,308	226	ERS4662530
S7	16,313,920	15,108,112	72,740	224	ERS4662409

highest genetic homology was with *A. phagocytophylum*. In addition to the known pathogens, sequences belonging to CMM (order Rickettsiales) were detected in high abundance in both ticks. CMM is an intra-mitochondrial bacterium present in female *I. ricinus* ticks, while its prevalence in males is lower (Sassera et al., 2006). It is hypothesized that the bacterium provides *I. ricinus* with metabolites or vitamins, modulates the immune response and improves cellular respiration of the ticks during blood feeding (Stavru et al., 2020). It has been reported that abundant bacterial endosymbionts, such as CMM, limit the effectiveness of 16S rRNA NGS by masking less abundant bacteria, including pathogens; therefore, a strategy using specific blocking primers that inhibit endosymbiont 16S amplification during PCR has been proposed (Gofton et al., 2015).

Two male *Rh. bursa* ticks (S3 and S4) were included in the present study. *R. massiliae* which is commonly detected in this tick species was detected in one tick (S4), together with *Anaplasma* spp., with sequences presenting highest similarity to *A. ovis*, which coincides with the results of the conventional PCR. A low percentage of CMM was also detected in that tick. Although CMM is associated mainly with *I. ricinus* ticks, it has been detected in several other tick species, including *Rh. bursa*. In a study in Italy the prevalence of CMM was 33% in females and 13% in males (Epis et al., 2008). In addition to the above bacteria, RNA sequences related to gamma-proteobacteria belonging to *Coxiella* spp. were detected both ticks. The low number of reads did not allow the identification of these bacteria at species level. However, BLAST analysis and results of the previous study showed that they were Coxiella endosymbionts, which are very common in *Rh. bursa* ticks (Raele et al., 2015).

Tick-borne pathogens were not detected in the *Rh. sanguineous, D. marginatus,* and *H. parva* ticks (S5–S7), while high abundance of *Coxiella* endosymbiont sequences were present in S5 and S7.

There was no evidence of *Coxiella pneumoniae*, *Bartonella, Ehrlichia Borrelia* and *Francicella* sequences in any of the analyzed ticks. A plethora of additional bacteria were detected in all ticks, which might be acquired either by the host (from the host's skin or ingested by ticks during host

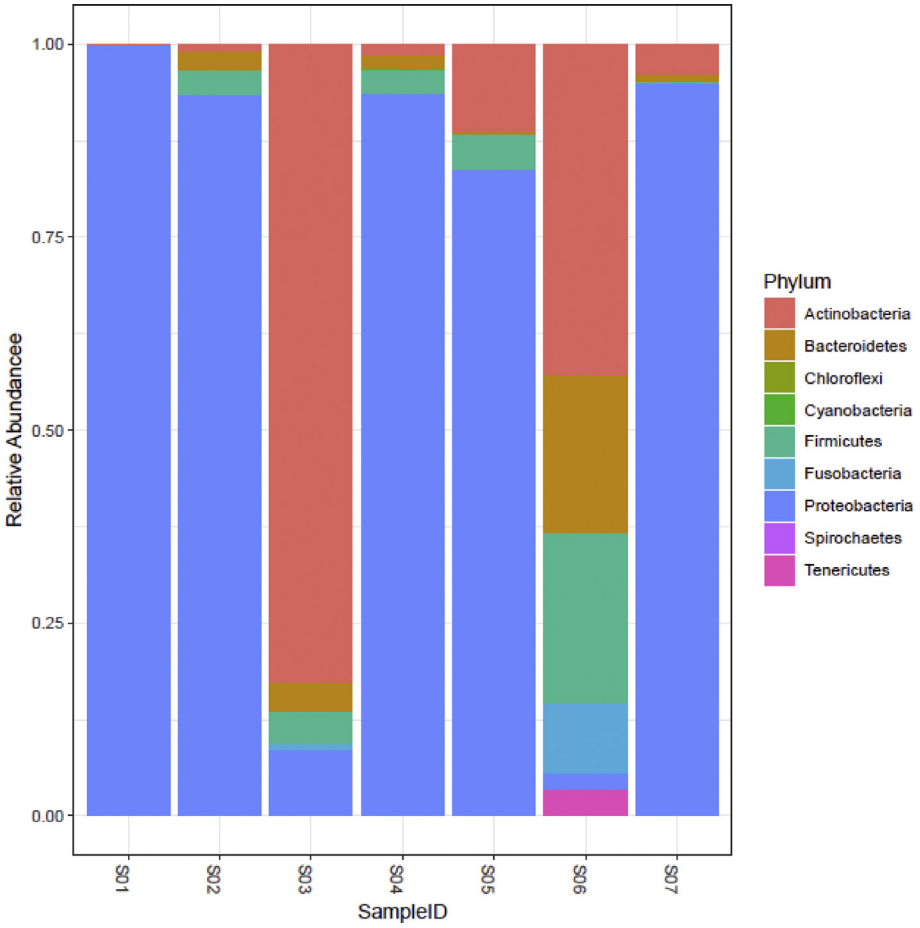

Figure 1. Relative abundance of bacterial phyla per individual ticks of the study.

Table 3. The most abundant bacteria detected in ticks: A. at family level B. at genus level.

A.			
Family (Phylum)	Number of ticks	Total abundance	Percentage
"*Candidatus* Midichloriaceae" (Proteobacteria)	5	441553	44.22
Rickettsiaceae (Proteobacteria)	3	158313	15.86
Corynebacteriaceae (Actinobacteria)	7	136297	13.65
Coxiellaceae (Proteobacteria)	7	115103	11.53
Porphyromonadaceae (Bacteroidetes)	4	20308	2.03
Prevotellaceae (Bacteroidetes)	4	12633	1.27
Clostridiales Family XI. Incertae Sedis (Firmicutes)	4	10587	1.06
Anaplasmataceae (Proteobacteria)	5	9910	0.99
Fusobacteriaceae (Fusobacteria)	2	9520	0.95
Enterobacteriaceae (Proteobacteria)	4	8565	0.86
Streptococcaceae (Firmicutes)	4	8301	0.83
B.			
Genera (Phylum)			
"*Candidatus* Midichloria" (Proteobacteria)	5	440490	48.47
Rickettsia (Proteobacteria)	3	157893	17.37
Corynebacterium (Actinobacteria)	7	122020	13.43
Coxiella (Proteobacteria)	7	86377	9.50
Prevotella (Bacteroidetes)	3	11890	1.31
Fusobacterium (Fusobacteria)	2	9520	1.05
Anaplasma (Proteobacteria)	4	8566	0.94
Streptococcus (Firmicutes)	4	8037	0.88
Porphyromonas (Bacteroidetes)	4	6188	0.68
Propionibacterium (Actinobacteria)	3	5609	0.62

feeding) or from the environment during the life cycle of the tick in nature.

In conclusion, a great variability was seen in the microbiome profile among ticks, which may reflect differences in the tick species, or host animal species and health status, or even geographic location. It was shown that all tick-borne bacteria previously detected by conventional PCRs were detected also using 16S rRNA NGS, while *Anaplasma* spp. was detected by NGS in one tick which was not detected by PCR. Besides the tick-borne pathogens, a plethora of additional bacteria constituting the tick bacteriome were detected. In general, 16S rRNA NGS provides a

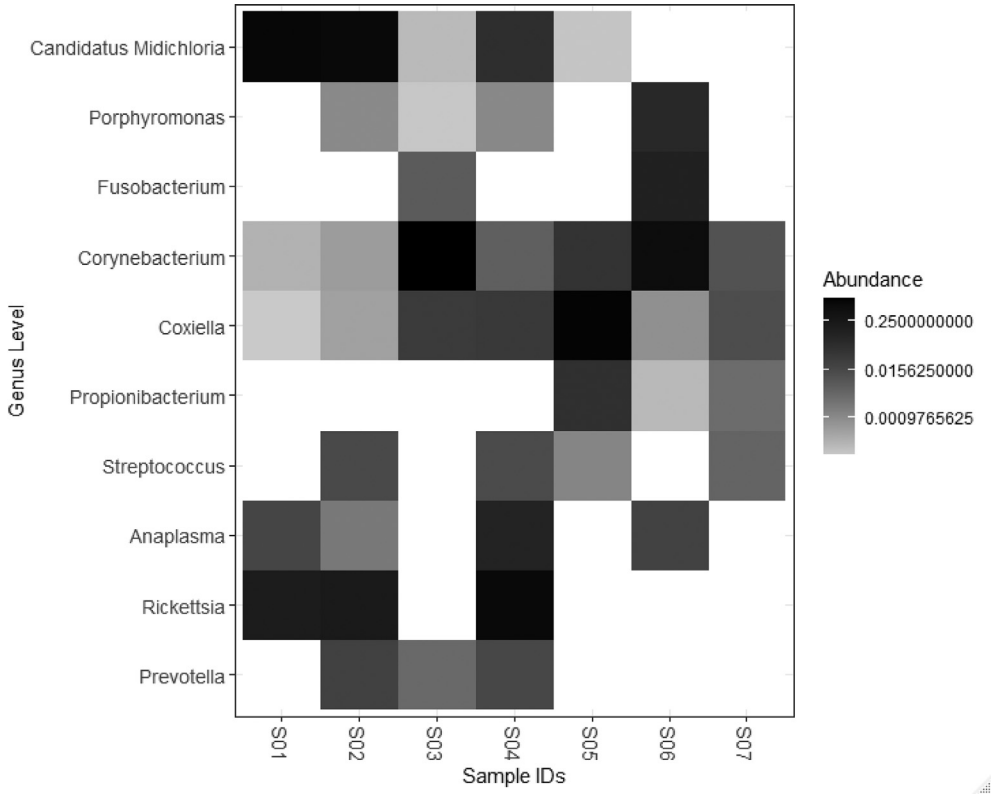

Figure 2. Genus-level heatmap plot per individual tick of the study.

Table 4. Genus-level abundance levels for individual ticks; the respective phyla, classes, orders, and families are seen in the first column.

Phylum/Class/Order/Family	Genus	Abundance	S1	S2	S3	S4	S5	S6	S7
Proteobacteria/Alphaproteobacteria/Rickettsiales/"*C.* Midichloriaceae"	"*Canditatus* Midichloria"	440490	80495	357703	17	2262	13	0	0
Proteobacteria/Alphaproteobacteria/Rickettsiales/*Rickettsiaceae*	*Rickettsia*	157893	19115	112891	0	25887	0	0	0
Actinobacteria/Actinobacteria Actinomycetales/*Corynebacteriaceae*	*Corynebacterium*	122020	26	263	78601	196	4543	38360	31
Proteobacteria/Gammaproteobacteria/Legionellales/*Coxiellaceae*	*Coxiella*	111579	12	224	2669	1217	82163	52	25242
Bacteroidetes/Bacteroidia/Bacteroidales/*Prevotellaceae*	*Prevotella*	11890	0	10988	315	587	0	0	0
Fusobacteria/Fusobacteriia/Fusobacteriales/*Fusobacteriaceae*	*Fusobacterium*	9520	0	0	582	0	0	8938	0
Proteobacteria/Alphaproteobacteria/Rickettsiales/*Anaplasmataceae*	*Anaplasma*	8566	1815	991	0	4384	0	1376	0
Firmicutes/Bacilli/Lactobacillales/*Streptococcaceae*	*Streptococcus*	8037	0	7413	0	491	119	0	14
Bacteroidetes/Bacteroidia/Bacteroidales/*Porphyromonadaceae*	*Porphyromonas*	6188	0	508	11	38	0	5631	0
Actinobacteria/Actinobacteria/Actinomycetales/*Propionibacteriaceae*	*Propionibacterium*	5609	0	0	0	0	5586	13	10

broad spectrum of bacteria present in ticks; then, targeted PCRs can be applied to identify the bacteria at species level, while whole genome sequences of the bacteria of interest can be obtained by additional NGS methods.

Limitations of the study were that the ticks were collected from animals and not from the field and that the number of tested ticks was low. Due to the small sample size, no conclusions can be made about the differences between the sites and tick species. However, this is a preliminary study on the application of 16s rRNA NGS approach to explore tick microbiome. 16S rRNA NGS is a powerful tool to elucidate the tick microbiome patterns in order to enable studies on interactions between pathogens, endosymbionts, ticks and their hosts. Furthermore, knowledge of tick microbiome in a region can improve the strategies for prevention and control of tick-borne diseases.

Declarations

Author contribution statement

Anna Papa: Conceived and designed the experiments; Wrote the paper.

Katerina Tsioka, Elpida Papadopoulou: Performed the experiments.

Maria-Antonia Daskou, Fani Minti, Ageliki Melidou: Analyzed and interpreted the data.

Nektarios Giadinis: Contributed reagents, materials, analysis tools or data.

Funding statement

This work was supported by the EU Horizon 2020 research and innovation programme COMPARE (grant agreement No 643476).

Competing interest statement

The authors declare no conflicts of interest.

Additional information

No additional information is available for this paper.

References

Andreotti, R., Perez de Leon, A.A., Dowd, S.E., Guerrero, F.D., Bendele, K.G., Scoles, G.A., 2011. Assessment of bacterial diversity in the cattle tick *Rhipicephalus (Boophilus) microplus* through tag-encoded pyrosequencing. BMC Microbiol. 11, 6.

Chaligiannis, I., Musella, V., Rinaldi, L., Cringoli, G., de la Fuente, J., Papa, A., Sotiraki, S., 2016. Species diversity and spatial distribution of ixodid ticks on small ruminants in Greece. Parasitol. Res. 115, 4673–4680.

Couper, L., Swei, A., 2018. Tick microbiome characterization by next-generation 16S rRNA amplicon sequencing. JoVE.

Cross, S.T., Kapuscinski, M.L., Perino, J., Maertens, B.L., Weger-Lucarelli, J., Ebel, G.D., Stenglein, M.D., 2018. Co-infection patterns in individual *Ixodes scapularis* ticks reveal associations between viral, eukaryotic and bacterial microorganisms. Viruses 10.

de la Fuente, J., Antunes, S., Bonnet, S., Cabezas-Cruz, A., Domingos, A.G., Estrada-Pena, A., Johnson, N., Kocan, K.M., Mansfield, K.L., et al., 2017. Tick-pathogen interactions and vector competence: identification of molecular drivers for tick-borne diseases. Front. Cell Infect. Microbiol. 7, 114.

Epis, S., Sassera, D., Beninati, T., Lo, N., Beati, L., Piesman, J., Rinaldi, L., McCoy, K.D., Torina, A., et al., 2008. Midichloria mitochondrii is widespread in hard ticks (*Ixodidae*) and resides in the mitochondria of phylogenetically diverse species. Parasitology 135, 485–494.

Gofton, A.W., Oskam, C.L., Lo, N., Beninati, T., Wei, H., McCarl, V., Murray, D.C., Paparini, A., Greay, T.L., et al., 2015. Inhibition of the endosymbiont "*Candidatus* Midichloria mitochondrii" during 16S rRNA gene profiling reveals potential pathogens in Ixodes ticks from Australia. Parasites Vectors 8, 345.

Greay, T.L., Gofton, A.W., Paparini, A., Ryan, U.M., Oskam, C.L., Irwin, P.J., 2018. Recent insights into the tick microbiome gained through next-generation sequencing. Parasites Vectors 11, 12.

McMurdie, P.J., Holmes, S., 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PloS One 8, e61217.

Oksanen, J., Blanchet, F.J., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., et al., 2016. Vegan: Community Ecol. Package. R Package Version 2.4-2.

Papa, A., Tsioka, K., Kontana, A., Papadopoulos, C., Giadinis, N., 2017. Bacterial pathogens and endosymbionts in ticks. Ticks Tick Borne Dis 8, 31–35.

Parola, P., Raoult, D., 2001. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. Clin. Infect. Dis. 32, 897–928.

Raele, D.A., Galante, D., Pugliese, N., De Simone, E., Cafiero, M.A., 2015. *Coxiella*-like endosymbiont associated to the "Anatolian brown tick" *Rhipicephalus bursa* in Southern Italy. Microb. Infect. 17, 799–805.

Roux, V., Raoult, D., 1995. Phylogenetic analysis of the genus *Rickettsia* by 16S rDNA sequencing. Res. Microbiol. 146, 385–396.

Sassera, D., Beninati, T., Bandi, C., Bouman, E.A., Sacchi, L., Fabbi, M., Lo, N., 2006. '*Candidatus* Midichloria mitochondrii', an endosymbiont of the tick Ixodes ricinus with a unique intramitochondrial lifestyle. Int. J. Syst. Evol. Microbiol. 56, 2535–2540.

Stavru, F., Riemer, J., Jex, A., Sassera, D., 2020. When bacteria meet mitochondria: the strange case of the tick symbiont Midichloria mitochondrii (dagger). Cell Microbiol. 22, e13189.

Wernegreen, J.J., 2012. Endosymbiosis. Curr. Biol. 22, R555–R561.

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York.

Wikel, S.K., 2018. Ticks and Tick-Borne Infections: complex ecology, agents, and host interactions. Vet Sci 5.