Tick-borne pathogens identified in *Hyalomma marginatum* (Acari: Ixodidae) collected from various vertebrate hosts in the South of France.

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ABSTRACT

Hyalomma marginatum is a common ectoparasitic tick of ungulates, lagomorphs, insectivores, ground-foraging birds, observed in Corsica for decades, but whose permanent establishment in mainland France is very recent. This species is known to be one of the main vectors of the Crimean-Congo Hemorrhagic Fever virus, but also of various parasitic, bacterial or viral pathogens. In this study, we investigated the molecular infection rates of numerous tick-borne pathogens in ticks mainly sampled on horses, and occasionally on other animal species, from the French Mediterranean rim and Corsica between 2016 and 2020. In total, 1, 195 DNA and RNA purified from individual tick or pools of ticks were screened for 26 microbial genera or species (viruses, bacteria and parasites), using a high-throughput microfluidic real-time PCR system (BioMark™ dynamic array system, Standard Biotools). For individual ticks and pooled ones, respectively, the most prevalent tick-borne microorganisms were *Francisella*-like endosymbionts at 97.0% and 96.8%, followed by *Rickettsia aeschlimannii* (76.4% and 96.4%), *Theileria* spp. and *Theileria equi* (3,5% and 0%; 1,9% and 5,8%), *Anaplasma phagocytophilum* (3.7% and 6.7%), and West Nile virus (0.1% and 0.4%). *Babesia occultans* (0.9%), *Ehrlichia minasensis* (0.3%), and*Coxiella*-like endosymbionts (0.1%) were only detected in individual ticks. Our study provides an overview of the diversity of microorganisms and tick-borne pathogens detected in the invasive tick *H. marginatum* in

Mediterranean France. Our results question the possible distribution of tick-borne pathogens in Corsica and the continental departments of the Mediterranean rim. Our study opens up new research perspectives on the epidemiology of tick-borne pathogens carried by *H. marginatum* and on the associated public and veterinary health risks.

Keywords: Tick; *Hyalomma marginatum*; tick-borne pathogens; endosymbionts, microfluidic PCR

Introduction

Worldwide, ticks are considered to be the first vectors of pathogens responsible for animal diseases and second behind mosquitoes for those affecting humans (Jongejan et Uilenberg 2004; Colwell, Dantas-Torres, et Otranto 2011). In France, about forty species of ticks are identified on the territory, most of them transmitting pathogens responsible for important public and veterinary health problems (Boulanger et McCoy 2017; PÉREZ-EID 2007). In particular, this includes *Ixodes ricinus* which is the main vector of the agents of Lyme borreliosis, *Borrelia burgdorferi* sensu lato, or ticks from the genera *Dermacentor* or *Rhipicephalus* responsible for the transmission of equine piroplasmosis (Gray 1991; Nadal, Bonnet, et Marsot 2022). Recently, Vial et al. (2016) provided strong evidence for the presence of reproducing populations of the exotic tick *Hyalomma marginatum* in parts of southern France. While this tick species has been present in Corsica (a Mediterranean island of southern France) for several decades, it seems to be now established on the Mediterranean coastline of mainland in many areas such as Pyrénées-Orientales, Aude, Hérault, Gard, Var, Ardèche and Drôme (Bah et al. 2022). This tick has been also detected since many years in Spain (Palomar et al. 2016) and Italy (Satta et al. 2011), showing a possible continuum for the sanitary risks associated to *H. marginatum* in southwestern Europe. As a "generalist" tick, *H. marginatum* presents a large panel of vertebrate hosts, ranging from lagomorphs and birds for the immature stages to large domestic and wild ungulates for the adult ones (Bernard et al., 2022. This is a two-host tick, meaning that larvae and nymphs feed on the same individual, while the nymph detaches and moults into an adult that seeks out a new host for blood feeding. This diversity of hosts multiplies pathways for tick introduction through natural or human-driven animal movements and supposedly, increases occasions of pathogen transmission and possible spillover. Ticks of the genus *Hyalomma* are involved in the transmission of many human and animal pathogens in Eurasia and Africa (Bonnet et al. 2023). They can in particular carry parasites and bacteria such as *Theileria* spp., *Babesia* spp. and *Anaplasma* spp., responsible for piroplasmosis or other clinical signs (e.g. hyperthermia, asthenia, abortion...) in cattle or sheep (15, 16,17). Other infectious bacteria such as *Coxiella burnetii*, and *Rickettsia* spp. including *R aeschlimannii* (Bakheit et al. 2012; Kilicoglu et al. 2020; Orkun 2022), or viruses such as Bhanja (Genus Bandavirus) and West Nile (Genus Flavivirus) (Formosinho et Santos-Silva 2006; Kolodziejek et al. 2014), can be carried by *H. marginatum*. Additionally, Orthonairoviruses such as the emerging and highly contagious Crimean Congo Hemorrhagic Fever virus (CCHFV) (Bakheit et al. 2012) can be transmitted by this tick spcies. In the Mediterranean basin, two species of *Hyalomma* (*H. marginatum* and *H. anatolicum*) are used to be considered as the main vectors of CCHFV (Perveen et Khan 2022). However, viral RNA has been recently detected in *H. lusitanicum* collected from deer and wild boar in Spain, in higher proportion than in *H. marginatum*, suggesting major role of this additional tick in CCHFV transmission in this country (Moraga-Fernández et al. 2021). In France, based on our knowledge on local tick communities, we considered *H. marginatum* as the main candidate tick species likely to transmit CCHFV (Bernard et al. 2022). Considering the large geographic distribution of *H. marginatum*, from Spain to India and North Africa (Fernández-Ruiz et Estrada-Peña 2021), this questions about local adaptations in the abilities developed by this tick species to maintain and transmit all, or part of the pre-cited pathogens.

The objective of this study was thus to describe the panel of pathogens and/or endosymbiotic microorganisms in *H. marginatum* ticks locally collected in the South of France, especially mainlandThis work was part of a national surveillance program developed a few years ago to assess the risk of emergence of exotic diseases including tick-borne diseases in France, under the aegis of the French Ministry of Agriculture. In Corsica, first investigations were recently conducted on tick species collected from different hosts, especially cattle, and reported in *H. marginatum* high prevalence of *Francisella*-like endosymbionts, *Ri. aeschlimannii* and in a less extend *Candidatus Ri. Barberiae*, as well as the presence of *Anaplasma marginale* and *A. phagocytophylum* (V. Cicculli et al. 2019; Grech-Angelini et al. 2020). These studies also allowed the detection of new viruses in *H. marginatum*, for example a Pseudocowpox virus of which the zoonotic potential remains unknown (V. Cicculli et al. 2019). On the mainland where *H. marginatum* was not yet established only 15 years ago, such investigation has never been done. Keeping in mind that genomic detection of pathogens does not imply a mandatory vector competence of the tick for the considered microorganisms (Bonnet et al. 2023), the aim of this study was to know whether *H. marginatum* ticks arrived with their native associated pathogens usually described in endemic Mediterranean areas including Corsica, and may result in the introduction of new pathogens in France. Another alternative

would be that *H. marginatum* can become a new vector for local French tick-borne pathogens already present on the territory, with the risk of changing the local transmission dynamics of such pathogens due to its distinct seasonal activity from other local tick species.

Methods

Study area, period and hosts for tick collection

Among all the *H. marginatum* ticks analyzed in this study, 1,711 came from a large-scale tick sampling that was conducted on horses (the likely host of *H. marginatum* adult stages), at spring (the activity period of *H. marginatum* adult stages), in southern France (mainland and Corsica), between 2016 and 2020. This was carried out to determine and explain the distribution of the newly established tick *H. marginatum* on the French territory (Bah et al. 2022). Fourteen French departments forming a link from Spain to Italy, which are two countries where *H. marginatum* is known to be already present, plus the two departments from Corsica were sampled. *H. marginatum* ticks were detected in 56 and 38 equestrian structures in mainland France and in Corsica, respectively. On the mainland, a reference site in Pompignan, where *H. marginatum* is abundant and monitored for its dynamics of infestation on horses, was also sampled each year and 173 ticks from this site were finally included in this study. In Corsica, each year, the whole island was visited whereas on the mainland, most of *H. marginatum* were obtained in 2017, except for northern Gard and Ardèche that were investigated in 2019 (Figure 1). Among the 1,711 ticks, 900 collected in Corsica in 2019 and 2020 were grouped into 222 pools of 1 to 10 individuals according to different parameters of sex or hosts (Cicculli et al., 2022) and 811 were treated individually.

In Corsica, 118 ticks were also collected from cattle examined in three farms located in the northern part of the island, in 2019, and completing the tick monitoring carried out in slaughterhouses for tick-borne disease surveillance (Cicculli et al. 2019; Cicculli et al. 2022). Additionally, 7 and 37 *H. marginatum* ticks were obtained by punctual samplings in Hérault and Gard on wild boars in 2016 and birds in 2017, respectively. Ticks from birds were all immature stages, and were mostly associated to the resident avian fauna of Pompignan (Vial et al. 2016). Thus, taking all host species together, we worked on 973 ticks treated individually and 222 tick pools (1,195 samples).

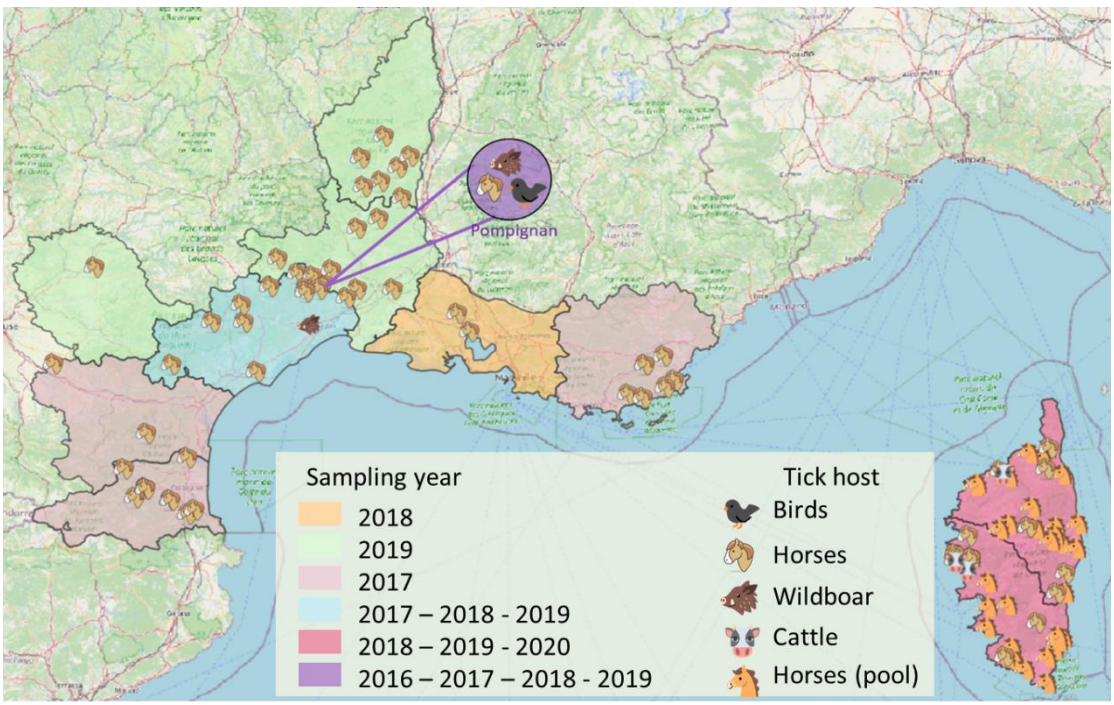

Figure 1: Study area overview : geographical scope of research. On this map, sites are represented by the host species from which the ticks were collected (horses, cattle, wild boars and birds).

During any tick samplings, animals were examined on the whole body and all ticks were collected. Back from the field, they were determined using morphological identification keys (PÉREZ-EID 2007), and sorted according to species, locality, host, gender (male/female), developmental stage (larva/nymph/adult), and engorgement status. They were then frozen at -80°C for later molecular analysis. As this study focused on sanitary risks associated to the newly established *H. marginatum*, only specimens from this species were screened for microorganisms.

DNA and RNA extraction

As we looked for DNA and RNA of microorganisms in all tick samples, their treatment for molecular analysis was the same.

After a first washing in bleach for 30 sec followed by three times in distilled water for 1 min, ticks were crushed individually with three steel beads (2.8mm, OZYME, France) in 300µL DMEM (Eurobio Scientific, France) supplemented with 10% fetal calf serum (Dutscher, France), using the homogenizer Precellys® 24 Dual (Bertin, France) at 5500 rpm for 40s. The processing was different for samples of horses coming from Corsica as, prior to be crushed, ticks were pooled by up to 10 individuals per pool, according to locality and developmental stage (Vincent Cicculli et al. 2022).

DNA was extracted using 100µl of the homogenate of crushed ticks according to the Genomic DNA from tissue kit (Macherey-Nagel, Germany) following the manufacturer's instructions. Total DNA per sample was eluted in 50 µl of rehydration solution and stored at −20◦C until further use (Michelet et al, 2014).

Total RNA was extracted from 100µl of the homogenate of crushed ticks using the NucleoSpin RNA kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Total RNA per sample was eluted in 50 µl of RNA free water and stored at −80◦C until further use (Gondard et al., 2018).

Detection of tick-borne microorganisms' DNA or RNA

The Reverse Transcription Master Mix kit (Standard Biotools, USA) was used on all samples to obtain cDNAs from RNA viruses. The reaction was performed in 5µL of final volume, including 1µL of Reverse Transcription Master Mix, 3µL of RNase-free ultrapure water, and 1µL of extracted RNA. A thermal cycler (Eppendorf, Germany) performed the RT cycles following the program: 25°C for 5 min, 42°C for 30 min and a final step at 85°C for 5 min for a total duration of 40 min.

A pre-amplification step was performed to pre-amplify the DNA/cDNA to increase the signal of the genetic material of the microorganisms compared to those of the tick. The PreAmp Master Mix kit (Standard Biotools, USA) was used. A 0.2X pool containing the primer pairs of the targeted pathogens was made upstream, to obtain a final primer concentration of 200 nM. For one sample, the mix was composed of 1 µL of PreAmp Master mix, 1.25 µl of the 0.2X pool, and 1.5 µl of ultra-pure water. Then 1.25µL of sample (DNA/cDNA mix) was added. A negative control (ultra-pure water) was added to each plate. PCR was then performed using a thermal cycler with the following program: 95°C for 2min, 14 cycles of two steps of 95°C for 15s and a final step of 4min at 60°C.

Then, for the real time microfluidic PCR, twenty-four sets of primers and probes were used to detect predefined microorganisms specifically targeted for this study (Table 1) (Michelet et al. 2014; Gondard et al. 2018). The BioMark™ real-time PCR system (Standard Biotools, USA) was used for high throughput microfluidic real-time PCR amplification using 48.48 dynamic arrays (Standard Biotools). These chips dispense 48 PCR mixes and 48 samples into individual wells, after which on-chip microfluidics assemble PCR reactions in individual chambers prior to thermal cycling resulting in 2,304 individual reactions (Michelet et al. 2014). In a single experiment, 47 individual ticks or tick pools samples (and one negative control) can be tested.

In order to develop the a priori approach to detect pathogens in ticks, we first generated a list of microorganisms (TBPs and endosymbionts) (Table 2) with the highest probability of being transmitted or carried by *H. marginatum*, according to data mining of the available literature. From two studies *(Bakheit et al. 2012; Bonnet et al. 2022)*, we identified several potentially *H. marginatum*-borne pathogens: protozoa such as *B. caballi* or *T. annulata*, bacteria such as *Ri. aechlimannii* and finally viruses such as Thogoto, Dhori and Bhanja viruses (RNA for these viruses has already been detected in ticks), in addition to CCHFV. We then identified microorganisms from scientific literature that mentioned the detection of pathogens in *H. marginatum*, which could be transmitted by this tick or just reflect the infectious status of the animal host on which the tick was collected, as shown in Corsica or other regions of the world: *A. phagocytophilum*, *A. marginale (Grech-Angelini et al. 2020)*, *E. minasensis (Vincent Cicculli et al. 2019)*, *T. equi (Rocafort-Ferrer et al. 2022)*, the West Nile Virus *(Formosinho et Santos-Silva 2006; Kolodziejek et al. 2014)*, the symbiotic form of *Coxiella burnetii* and *Francisella tularensis* : *Coxiella*-like endosymbiont CLE and *Francisella*-like endosymbiont FLE *(Kilicoglu et al. 2020; Selmi et al. 2019; Buysse et al. 2021; Demir et al. 2020)*. Then, some pathogens known to be transmitted by ticks of the genus *Hyalomma* but not necessarily *H. marginatum*, or even by other tick genera but with a likelihood to replicate in *Hyalomma* ticks, were also added to the chip: Alkhurma virus transmitted by *H. rufipes (Horton et al. 2016; Hoffman et al. 2018)*, as well as Nairobi sheep disease and Hazara viruses that are genetically close to CCHFV (Garrison *et al., 2020*, *Borrelia miyamotoi* and *Bartonella henselae* that circulate in France in other tick species or even other arthropoda such as fleas *(Cosson et al. 2014; Grech-Angelini et al. 2020)*.

Table 1: Tick-borne pathogens and endosymbionts targeted by the chip of the high-throughput microfluidic real-time PCR system developed specifically for the study.

Genus / Family	Species
Bacteria	
Anaplasma	*A. marginale*, *A. phagocytophilum*, *Anaplasma* spp.
Bartonella	*B. henselae*, *Bartonella* spp.
Borrelia	*B. miyamotoi*, *Borrelia* spp.
Coxiella	*Coxiella burnetii and Coxiella-like endosymbionts*
Ehrlichia	*Ehrlichia* spp.
Francisella	*F. tularensis*, *Francisella-like endosymbionts*
Rickettsia	*R. aeschlimannii*, *Rickettsia* spp.
Protozoan	
Babesia	*B. caballi*
Theileria	*T. equi*, *T. annulata*, *Theileria* spp.
Viruses	
Bunyaviridae	Nairobi sheep disease virus
Flaviviridae	West Nile Virus, Alkhurma virus
Nairoviridae	Hazara virus, CCHFV
Orthomyxoviridae	Thogoto virus, Dhori virus
Phenuiviridae	Bhanja virus

To confirm our results of positive detection of pathogens by the microfluidic chip in field samples, specific PCRs were performed individually on the corresponding samples with the following primers (Table 2). Conventional PCRs using primers targeting genes or regions other than those of the BioMark™ system were used. Amplicons were sequenced by Eurofins MWG Operon (Germany), and then assembled using BioEdit software (Ibis Biosciences, Carlsbad). An online BLAST (National Center for Biotechnology

Information) was used to compare results with published sequences listed in GenBank sequence databases.

Table 2: Primers used to confirm each pathogen detected by the microfluidic chip, through conventional and/or nested PCR.

Pathogen	Name	PCR type		Amorce name	Sequence
Anaplasma / Ehrlichia sp.	EHR	Nested	PCR1	EHR1	GAACGAACGCTGGCGGCAAGC
				EHR2	AGT AYC GRA CCA GAT AGC CGC
			PCR2	EHR3	TGCATAGGAATCTACCTAGTAG
				EHR2	AGT AYC GRA CCA GAT AGC CGC
	msp4	Conventionnal		MSP4AP5	ATG AAT TAC AGA GAA TTG CTT GTA GG
				MSP4AP3	TTA ATT GAA AGC AAA TCT TGC TCC TAT
Babesia / Theileria sp.	BTH	Nested	PCR1	BTH 18S 1st F	GTGAAACTGCGAATGGCTCATTAC
				BTH 18S 1st R	AAGTGATAAGGTTCACAAAACTTCCC
			PCR2	BTH 18S 2nd F	GGCTCATTACAACAGTTATAGTTTATTTG
				BTH 18S 2nd R	CGGTCCGAATAATTCACCGGAT
	babG	Conventionnal		BABGF2	GYY TTG TAA TTG GAA TGA TGG
				BABGR2	CCA AAG ACT TTG ATT TCT CTC
Borrelia sp.	FlaB	Nested	PCR1	FlaB280F	GCAGTTCARTCAGGTAACGG
				FlaRL	GCAATCATAGCCATTGCAGATTGT
			PCR2	flaB	GCATCAACTGTRGTTGTAACATTAACAGG
				FlaLL	ACATATTCAGATGCAGACAGAGGT
Rickettsia sp.	rsfg	Conventionnal		Rsfg877	GGGGGCCTGCTCACGGCGG
				Rsfg1258	ATTGCAAAAAGTACAGTGAACA
	rc.rompB	Nested	PCR1	Rc.rompB.4362p	GTCAGCGTTACTTCTTCGATGC
				Rc.rompB.4,836n	CCGTACTCCATCTTAGCATCAG
			PCR2	Rc.rompB.4,496p	CCAATGGCAGGACTTAGCTACT
				Rc.rompB.4,762n	AGGCTGGCTGATACACGGAGTAA

Statistical analysis

The statistical analysis was conducted using R software (version 4.2.1, R Core Team).

In order to investigate the potential difference in the presence of *Rickettsia* between male and female ticks, a chi-square test was performed. The chi-square test is a statistical method commonly used to assess associations between categorical variables. The variables of interest were the presence of *Rickettsia* (1,0) and the gender (male or female), and the test was conducted to determine if there was a statistically significant association between gender and the presence of *Rickettsia*.

Results

1. Identification of microorganisms detected in H. marginatum

DNA and RNA of ten of the targeted tick-borne microorganisms were identified in 1195 *H. marginatum* ticks (973 individual ticks and 222 pools of 1 to 10 ticks) collected mainly on horses in the South of France, especially from continental areas, using the microfluidic real-time PCR system as a first screening method and then specific confirmation with dedicated conventional PCRs and sequencing. Using the Fluidigm

method, we detected 2 ticks positive for *Borrelia* spp, 3 for *A. marginale*, 33 for *A. phagocytophilum*, 201 for *Anaplasma* spp, 3 for *Ehrlichia* spp, 878 for *Ri. aeschlimannii*, 957 for *Rickettsia* spp, 8 for *B. henselae*, 4 for *Bartonella* spp., 68 for *Theileria* spp, 22 for *T. equi*, 13 for West Nile Virus, 1 for CLE and 1 160 FLE for individual tick analysis. In terms of tick pools, the same method identified 19 positive pools for *Anaplasma* spp., 215 pools for *Ri. aeschlimannii*, 214 for *Rickettsia*. spp., 10 for *Theileria*. spp., 5 for *T. equi*, 216 for FLE and 3 in WNV. Except for the well-known CLE and FLE (and because the pathogenic forms *C. burnettii* and *F. tularensis* were never detected in this study), any other amplified microorganisms suspected to be pathogenic were sequenced to be confirmed.

For *Rickettsia* spp*.,* most of samples amplified specifically for *Ri. aeschlimannii* were also amplified in *Rickettsia spp.*, using the microfluigdim method. 18 amplicons (5 combining the two targets, 3 amplifying only *Ri. aeschlimannii* and 10 amplifying only *Rickettsia* spp.) were sequenced. Of the 10 readable sequences, all sequences confirmed the presence of *Ri. Aeschlimannii*. One of these sequences has been deposited on GenBank (AN: PP379722), showed 98.77% homology with a strain isolated from an *H. marginatum* found in England in 2019 (AN: MT365092) and 97.85% homology with another strain detected in Corsica in 2019 (AN: MK732478) in a *Rhipicephalus bursa* tick.

For *Anaplasma* spp., all samples amplifying *A. marginale* were also positive for *Anaplasma* spp., which was not necessarily the case for *A. phagocytophilum* where samples were positive for *A. phagocytophilum* but not *Anaplasma* spp*.*, and many samples were only amplified using primers targeting *Anaplasma* spp. 19 amplicons representative of the four cases (*A. marginale* + *Anaplasma* spp. / *A. phagocytophilum* only / *A. phagocytophilum* + *Anaplasma* spp. / *Anaplasma* spp. only) were sequenced, and only confirmed samples that combined the amplification of both specific and generic targets. The samples positive for *Anaplasma* spp. also identified *Devosia* spp. by sequencing confirmation PCR products which is an ubiquitous soil bacteria that is considered as a PCR cross contaminant. Finally, two *Anaplasma* species were confirmed: *A. phagocytophilum* and *A. marginale*. The sequence of *A. phagocytophilum* obtained (GenBank, AN: PP227262) showed 99% homology with the reference sequence from an *A. phagocytophilum* strain isolated in a *Rhipicephalus microplus* tick collected on a cow in Taiwan in 2021 (AN: OL690562.1) and 99.12% with a sequence from an *Ixodes ricinus*, in Hérault, France in 2010 (AN : OR426543). For *A. marginale*, the sequence obtained (GenBank, AN : PP218689) showed 96% homology with reference sequence from an *A. marginale* strain isolated in a *H. schulzei* tick collected in Iran in 2019 (AN: MK310488) and 95,27 % from a strain isolated in cattle in 2017 in spain (AN: MT664983).

For *Ehrlichia* spp*.,* two amplicons were sequenced and were confirmed as *E. minasensis*. The resulting sequence deposited in GenBank (AN: PP227261) showed 99% homology with the reference sequence NR_*148800.1* of a strain*,* which was isolated from the hemolymph of a *Riphicephalus microplus* tick from Brazil in 2019. Moreover, the sequence detected shows 98% homology with AN : JX629805 detected in *Riphicephalus (Boophilus) microplus* in 2016 of which the sequence detected in Corsica was 100% identical (Vincent Cicculli et al. 2019).

For *Theileria* spp*.*, most of samples that were amplified specifically for *T. equi*, were also amplified for *Theileria* spp*.*, except two samples only for *T.equi*, whereas other samples were only amplified for *Theileria* spp. No sample was amplified for *T. annulata*. 66 amplicons combing these three cases (*T. equi* only / *T. equi + Theileria* spp*.* / *Theileria* spp*.* only) were sequenced and confirmed the presence of *T. equi* for samples amplified for both targets as well as those amplified only for *T. equi*. All amplicons for *Theileria* spp. only produced sequences that could not allow species-specific identification despite repeated sequencing (unreadable sequence). For *T. equi,* the same sequence (GenBank, AN: PP227191) was obtained and showed 99% homology with the reference sequence from a *T. equi* strain isolated in Chile (AN: MT463613.1) in 2020 and 99,81% homology with a sequence from *Rhipicephalus bursa* in Corsica in 2019 (AN: MK732476.1).

Nine ticks detected positive for *Theileria* spp. only were finally confirmed by sequencing as infected by *Babesia occultans*. This ticks were only collected from cattle. The sequence was deposited in Genbank (AN: PP227192) and showed 99% homology with a sequence of *B. occultans* isolated from a *H. excavatum* tick collected on cattle in Turkey in 2022 (AN : OM066180.1).

One individual tick and one pool of ticks from Corsica were positive for the West Nile Virus. The amplification product was sequenced and deposited in Genbank (AN: XXXXXX) and showed 100% homology with the corresponding sequence in the complete genome detected from snowy owl in 2019 in Germany (AN : LR743458.1).

3. Prevalence of microorganisms in H. marginatum, host origin and spatial distribution

Results of individual and pooling detections are presented in Table 3.

FLE was the most prevalent microorganism, as detected in more than 95% of individual ticks and pools of ticks of *H. marginatum*. *Ri. aechlimannii* was detected in more than 75% of the analyzed individual ticks and 95% of pools of *H. marginatum*. Conversely, we only identified a CLE in one tick collected on a horse in Var. Then, prevalences of infection due to all the other targeted tick-borne pathogens ranged from 0.1 to 3.5% (up to 6.7% in pools). If we remove the associations between *Ri. aeschlimannii* that was highly prevalent and the other pathogens, the likelihood of coinfections in *H. marginatum* ticks seemed to be very low. In fact, only 3 coinfections were observed, two ticks carried both *B. occultans* and *A. marginale* and one tick carried both *E. minasensis* and *A. marginale*.

Although equine piroplasmosis is commonly reported in horses from the South of France (Rocafort-Ferrer et al. 2022) and ticks were mainly collected on horses, *T. equi* was detected in a very small number of ticks, 17 individual ticks from mainland France and 9 pools from Corsica, and no *B. caballi* was detected in the analyzed ticks. However, *B. occultans* was detected in nine individual ticks collected on cattle from Corsica. For bacteria, we evidenced two species of *Anaplasma* and one of *Erhlichia. A. marginale* was only detected in ticks collected on cattle in Corsica, while *A. phagocytophilum* was mainly found in ticks collected from horses but also on cattle and to a lesser extent in ticks collected on birds (n = 2/37). Birds were mainly infected by *Ri. aeschlimannii* (n = 15/37) (Table 3). *E. minasensis* was detected in four ticks, three from Corsican cattle and one from a horse on the mainland. Only one virus, the West Nile virus, was detected in one tick collected from a cow and one pool of ticks collected from a horse, both in Corsica (Table 3). *H. marginatum* ticks were all negative for CCHFV. No specific pathogens, except *Ri. aeschlimannii*, were found in wild boar ticks (n = 6/7). Except differences observed between ticks from horses versus cattle, and although *H. marginatum* ticks from other vertebrate hosts were highly underrepresented, their panel of microorganisms seemed to be quite similar to what was observed in horses.

Table 3: Microorganisms detected and confirmed in *H. marginatum* ticks, tested individually or by pools. The currently known or assumed pathogens are in black and the endosymbionts in grey.

	Individual ticks (N=973)			Pools of ticks (N=222)		
	Nb. detected	Proportion (%)	Host	Nb. detected	Proportion (%)	Host
Rickettsia aeschlimannii	743	76,4	B, C, H, W	214	95,1	H
Francisella-like endosymbiots	944	97,0	B, C, H, W	216	97,3	H
Anaplasma phagocytophilum	33	3,4	B, C, H	15	6,7	H
Anaplasma marginale	3	0,3	C	0	0	H
Ehrlichia minasensis	3	0,3	C, H	0	0	H
Coxiella-like endosymbiots	1	0,1	H	0	0	H
Theileria spp.	34	3,5	H	0	0	H
Theileria equi	17	1,8	H	13	5,8	H
Babesia occultans	9	0,9	C	0	0	H
West Nile Virus	1	0,1	C	1	0,4	H

B: birds, C: cattle, H: horses, W: wild boars

Using Chi-square test, except for *Ri. aeschlimannii* and *B. occultans* we did not observe a significant difference in the presence of each pathogen between males and females ticks (*Ri. aeschlimannii* : $\chi^2 = 10.28$, df = 1, $P = 0.001$; *A. phagocytophilum* : $\chi^2 = 0.46$ df = 1, $P = 0.49$; *A. marginale* : $\chi^2 = 1.37$, df = 1, $P = 0.24$; *E. minasensis* : $\chi^2 = 1.37$, df = 1, $P = 0.24$; *Theileria* spp. : $\chi^2 = 1.37$, df = 1, $P = 0.24$; *T. equi* : $\chi^2 = 0.001$, df = 1, $P = 0.97$; *B. occultans* : $\chi^2 = 4.14$, df = 1, $P = 0.04$). *Ri. aeschlimannii* seemed to be much more present in male than female ticks (n(M) = 388 ; n(F) = 340) and *B. occultans* in females (n(M) = 1 ; n(F) = 8). As explained in Material and methods, looking for differences between development stages or hosts on which ticks were collected was not applicable, due to misrepresentation of some categories.

By mapping results of tick infections (Figures 2), we did not observe spatial differences for most of the detected pathogens. This was confirmed statistically on data collected in 2017 only, which constituted the main part of the sampling. However, and keeping in mind that some host species such as cattle were not sampled in all areas, some pathogens were only detected in Corsica, such as *A. marginale* and *B. occultans*, as well as WNV. In addition, although *Ri. aechlimannii* was widely represented in ticks from the South of France, we noticed significant differences between geographical areas. Considering the geographical clusters previously described for the distribution of *H. marginatum* in southern continental France (Bah et al. 2022) the prevalence of *Ri. aeschlimannii* was significantly higher in Pyrénées-Orientales and Aude than in Hérault and Gard (Kruskal-Wallis, $\chi^2 = 8.6102$, df = 1, $P = 0,01$).

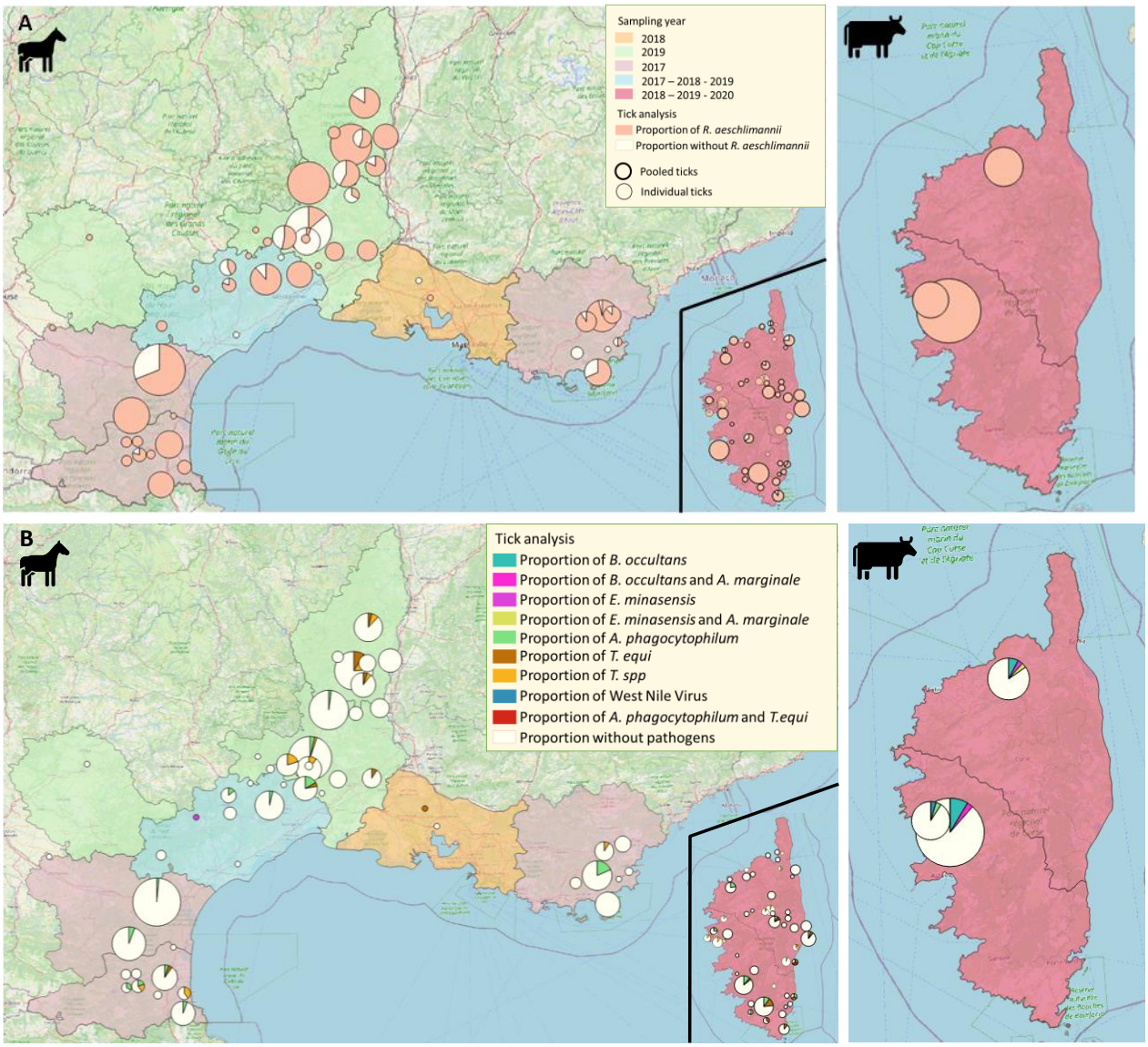

Figure 2: Distribution of *Rickettsia aeschlimannii (A)* in Ticks collected from Horses (Left) and Cattle (Right), and prevalence of various pathogens (B) in ticks from Horses (Left) and Cattle (Right) in each sampling site. The size of the circles is proportional to the number of ticks per site, the color of the department (in A and B) correspond to the samplig year.

Discussion

In this study, the microfluidic real time PCR technology was used for the detection of tick-borne microorganisms, including pathogens but also endosymbionts, in *H. marginatum* ticks collected mostly on horses and in a less extend on other animal species, in Corsica but also in the Mediterranean rim of mainland France where this species has recently settled (2016). The microfluidic method allowed the detection of eight known or suspected pathogens genera and species (namely *Ri. aeschlimannii*, *A. phagocytophilum*, *A. marginale*, *E. minasensis*, *Theileria* spp., *T. equi*, *B. occultans* and West Nile Virus) and two endosymbionts species (FLE and CLE) already known to circulate in France. Actually, the most abundant microorganisms detected were FLE and *Ri. aechlimannii*, whereas the prevalence of other microorganisms was low (0.1-6.7%).

The microfluidic method allows the high throughput targeted detection of defined parasites, bacteria and viruses, but may miss unexpected or new microorganisms that are present in the sample but not pre-listed as targeted candidates. This is questionable when investigating the panel of microorganisms that could be introduced by an invasive exotic tick species such as *H. marginatum*. This implies to identify, as comprehensively as possible, the range of microorganisms that are usually transmitted by, or at least detected in, such tick species in its original geographical areas, as it was done to develop and adapt the microfluidic array in this study. Our choice to target microorganism species but also larger genera and to confirm by sequencing allowed broadening our detection capacity, as it was the case for *B. occultans* confirmed in samples initially positive for *Theileria* spp. However, confirmation of the amplifications using sequencing were carried out only on a small part of samples, resulting in extrapolations for samples showing similar amplification pattern. This was the case of *Anaplasma* spp. and *A. phagocytophilum* for which, out of 19 samples sent for sequencing, only two sequences confirmed the presence of *A. phagocytophilum*. The other sequences obtained corresponded to *Devosia* spp., which is an ubiquitous soil bacterium, contaminating numerous samples and probably co-amplified by our PCR primers targeting Anaplasma spp. In this case, the samples that were positive for *A. phagocytophilum* on the chip were all confirmed by extrapolation compared with the sequences obtained for the two samples (which had the same profile on the chip). Nevertheless, the main advantage of this high-throughput method is its capacity to screen a large number of tick samples, for different microorganisms in parallel, with a very small amount of material allowing detection at the individual scale. In our case, this permitted to provide for the first time the panel of microorganisms encountered in 973 individual *H. marginatum* ticks and 900 in the 222 tick spools collected in 9 French departments, from Spain to Italian borders and Corsica island. This method also generated first prevalence estimates of microorganisms (including TBPs) carried by *H. marginatum,* depending on the animal hosts on which they were collected. In some cases, by crossing ecological and epidemiological information on tick-host-pathogen associations considered in our study, this allowed to provide first hypotheses on the role of *H. marginatum* as a vector for local pathogens. However, as *H. marginatum* is a hunting tick, all the ticks included in our study were collected on animals during their blood feeding. This consequently questions on the origin of microorganisms' DNA or RNA detected in ticks, namely the ticks as arthropod reservoirs or the infected animal hosts via the remaining blood meal in the tick midgut, which may change our conclusions on tick vector competence. Although experimental studies are scarce, especially for some microorganisms requiring high biosafety levels, only the experimental validation of the ability of a tick species to acquire a pathogen on an infected host, to allow its replication or persistence, and to transmit it to a new naïve host is a formal evidence of vector competence for a given pathogen.

Francisella-like and *Coxiella*-like endosymbionts detected in our study are non-pathogenic mutualistic and/or commensal microbes, which play a key role in the tick's developmental process and pathogen transmission (Buysse et al. 2021; Hussain et al. 2022). In our study, about 97% of *H. marginatum* were infected by FLE, and only one tick was positive for CLE, which is coherent with previous observations that FLE outcompetes CLE in ixodid ticks (Kumar et al. 2022). High prevalence of FLE in *H. marginatum* ticks

was previously observed, as it seems to be an ancestral primary endosymbiotic organism (mandatory to tick survival), being involved in nutritional symbiotic interactions including folate and riboflavin biosynthesis pathways plus biotin biosynthesis that are crucial for tick metabolism (Buysse et al. 2021)**.** Deprivation of their nutritional symbionts induced in ticks a complete stop of growth and moulting together with lethal physical abnormalities, which can be fully restored by exogenous supplementation with B vitamins. B vitamins are present at low concentrations in the blood compared to the amount required for arthropods development (Zhong, Jasinskas, et Barbour 2007; Duron et al. 2018; Serrato-Salas et Gendrin 2023), resulting in the need of production by endosymbionts.

The second most abundant bacterium carried by *H. marginatum* ticks was *Ri. aeschlimannii,* detected in 75% of individual ticks and 95% of tick pools. It was reported that *Ri. aeschlimannii* infections in humans can cause spotted fever, in North Africa and South Africa, and in 2010 a first case occurred in Southern Europe, in a Greek patient (Germanakis et al. 2013), and two other cases were reported in Italy in 2016 and in Germany in 2019 (44; https://www.vidal.fr/actualites/23772-cas-suspect-de-fievre-tachetee-a-rickettsia-aeschlimannii-en-allemagne.html). *Ri. aeschlimannii* was first isolated from *H. marginatum* in 1997 from Morocco (Beati et al. 1997), and is now regularly reported in ticks from the whole Mediterranean area (Parola et al. 2014) as in Corsica, where Grech-Angelini et al (2020) have detected 100% of the pools of *H. marginatum* positive to *Ri. aeschlimannii*. In the latter case, the pool analysis was able to increase the estimated prevalence of infection compared with our analyses of individual ticks, which showed 76.4% prevalence of infection. For the analysis of pooled samples in our study, two were negative, and each contained a single tick. Given the high infestation rate of this pathogen in continental or Corsican ticks, and the pathogenicity of this bacterium, the risk of human exposure to *Ri. aeschlimannii* could be very high. In Corsica, it has been hypothesized that the strong presence of this bacterium could explain human cases of tick-borne spotted fever (Grech-Angelini et al. 2020). On the other hand, in mainland France, human cases of tick-borne spotted fever have not been reported so far, and this questions about both the frequency of H. marginatum contacts with humans and the pathogenic potential of *Ri. aeschlimanii* found in *H. marginatum* ticks. *Ri. aeschlimannii* can be transovarially transmitted from *H. marginatum* ticks to their offspring, what strengthens the notion that *H. marginatum* may act as a reservoir for this bacterium (Matsumoto et al. 2004; Azagi et al. 2017). Considering the very high prevalence of *Ri. aeschlimannii* compared to any other pathogenic bacteria detected in *H. marginatum*, an alternative hypothesis could be its role as a secondary symbiont of *H. marginatum* ticks, which could regulate development of other infectious *Rickettsia* similarly to *Rickettsia peacockii* in *Dermacentor andersoni* (Baldridge et al. 2004). Contrary to a primary symbiont that is virtually fixed in the tick population because it is vital to them, secondary symbionts are not essential and may just give to ticks a higher selective value. At this stage, it is impossible to conclude on the role of *Ri. aeschlimannii* and further investigations are necessary, such as its detection specifically in tick salivary glands to conclude on its ability to be horizontally transmitted through tick bite and experimental trials of removal to describe its potential role for the tick metabolism.

Apart from *Ri. aeschlimannii*, bacterial DNA detected in *H. marginatum* was mostly represented by the genus *Anaplasma*, namely *A. phagocytophilum* and *A. marginale, remaining* both at low levels (3,7% in individual ticks, 6,7 % in pools for *A. phagocytophilum* and less than 1% of individual ticks for *A. marginale*). *A. phagocytophilum* is a zoonotic obligate intracellular bacterium causing Tick-Borne Fever in cattle and sheep, Human Granulocytic Anaplasmosis (HGA) in humans, and equine ehrlichiosis (Rikihisa 2011). This TBP has been reported in several countries in Europe (Matsumoto et al. 2006). There have been HGA cases reported in mainland France, with a first case identified in 2003 and others reported later but infrequently (Edouard et al. 2012). Human cases have also occurred in continental Italy (Ruscio et Cinco 2003). A recent study showed that in the French Pays Basque, 22.4% of collected *Rhipicephalus bursa* ticks contained *A. phagocytophilum* DNA (Dahmani et al. 2017). This is not the first time that this bacterium has been identified in *H. marginatum*, as one tick was found infected in Israel in 2007 (Keysary et al. 2007), and three in Tunisia in 2012 (M'ghirbi et al. 2012). As observed in our study, prevalence in ticks was always low (inferior to 3,5%), while the bacterium was highly present in cattle (Woldehiwet 2006) and horses (67% of positive horses, (M'ghirbi et al. 2012)), which are the likely hosts of *H. marginatum* adult stages. If ticks contracted the bacterium during their blood meal, there would supposedly be a larger proportion of positive ticks, and this allows us to assume a potential role of *H. marginatum* as vector or resevoir, a hypothesis that needs to be further investigated. Some tick species (*Dermacentor albipictus, Rh. annulatus*) transmit this pathogen trans-ovarially. This transmission has been estimated between 10 and 40% in *D.*

albipictus. In the absence of information on the vector competence of *H. marginatum* for *A. phagocytophilum* and in view of our results, we can say that *H. marginatum* may be involved in the transmission of this bacterium. Additionally to *A. phagocytophilum*, *A. marginale* DNA was found in three *H. marginatum* ticks collected from cattle in Corsica, which is consistent with its etiologic role in the erythrocytic bovine anaplasmosis that can affect various species of domestic and wild ruminants, including cattle (Aubry et Geale 2011), and that is largely distributed in France. This bacterium has been already reported in ticks from Corsica (Dahmani et al. 2017; V. Cicculli et al. 2019), including *H. marginatum* (Grech-Angelini et al. 2020). Although not considered as a primary vector of this bacterium (Atif 2015), and because we only found infected ticks when they were collected from cattle, it appears that the presence of this pathogen in our ticks could be the reflect of their host's infection. Another bacterium detected in *H. marginatum* was *E. minasensis.* In 2014, it was found to naturally infect cattle in Brazil and cause clinical ehrlichiosis in an experimentally infected calf (Aguiar et al. 2014). The distribution of *E. minasensis* is not restricted to the American continent (Gajadhar et al. 2010; Cruz et al. 2012; Aguiar et al. 2014), and this bacterium was reported recently in Corsica island (Vincent Cicculli et al. 2019), in Pakistan (Rehman et al. 2019), Ethiopia (Hailemariam et al. 2017), South Africa (Iweriebor et al. 2017) and Israel (Thomson et al. 2018). *E. minasensis* infects not only bovines (Gajadhar et al. 2010; Aguiar et al. 2014; Hailemariam et al. 2017), cervids (Lobanov et al. 2012) or dogs (Thomson et al. 2018), but also horses (Muraro et al. 2021). Although the vector competence of ticks for this microorganism remains to be tested, *E. minasensis* has already been identified in several tick species including *R. microplus* (Cruz et al. 2012; Carvalho et al. 2016), *R. appendiculatus* (Iweriebor et al. 2017) and *H. anatolicum* (Rehman et al. 2019). In 2019, Cicculli et al. (2019) identified this bacterium in 3.7% of *H. marginatum* collected from cattle in Corsica. In our study, *E. minasensis* was detected in few ticks, two from cattle in Corsica and one from a horse in mainland France (Hérault department). The three ticks were collected fully engorged and still attached to their host. The role of *H. marginatum* in the transmission of *E. minasensis* remains unknown. Nevertheless, despite very low observed prevalence, we cannot exclude but only hypothesize a vertical transmission to immature stages of *H. marginatum* or horizontal transmission from infected hosts or through co-feeding with other ticks, insuring possible maintenance and transmission of the bacterium.

Concerning protozoan microorganisms, DNA detected in *H. marginatum* was mostly represented by the genus *Theileria*. On one hand, there is *T. equi*, which is a causative agent of equine piroplasmosis (EP), which can also be due to *B. caballi* (not detected in this study) (Wise et al. 2013). *T. equi* infects wild and domestic equines worldwide, and are enzootic in many countries including several European countries bordering the Mediterranean sea such as France (Tirosh-Levy et al. 2020). The parasite is transmitted by hard tick species belonging to several genera including *Hyalomma, Dermacentor, Rhipicephalus, Amblyomma,* and *Haemaphysalis* (Scoles et Ueti 2015; Tirosh-Levy et al. 2020). This is not the first time that this apicomplexan parasite has been identified in *H. marginatum* in France, as 43% of ticks collected previously on horses in Camargue were found positive in 2022 (Rocafort-Ferrer et al. 2022). EP is known to be widely distributed in France, particularly in the South (Rocafort-Ferrer et al. 2022). If the majority of horses on which ticks engorged were positive and ticks could be infected *via* the blood meal, then they would have been almost all positive. However, we detected a few *T. equi* positive samples, despite the fact that many horses in the South of France are asymptomatic carriers of this parasite (Rocafort-Ferrer et al. 2022). This result may be explained by the fact that the areas sampled are not of high parasite circulation contrary to Camargue where the prevalence of *T. equi* is high (Rocafort-Ferrer et al. 2022), or by the fact that the primers used are not those that enable this parasite to be detected more widely or that *H. marginatum* is not a vector for this parasite. This avenue remains to be explored, and we will need to test again our samples with new primers to find out whether the prevalence of infection is increasing. However, this is not the only case where the prevalence of infection in horses was high although the prevalence of infection in ticks remained low. This was shown by Nadal (Nadal 2019), who found 50% infected horses and only 3% positive ticks. Additionally, as we did not detect any ticks positive for *B. caballi*, we can assume that whether this pathogen is not circulating or no longer circulating in the South of France, or that the primers used for PCR amplification also failed to detect it. We thus assume that few to no horses were actually infected by *B. caballi* in our sampling areas, which is commonly observed (Rocafort-Ferrer et al. 2022). On the other hand, About 5% of our collected ticks were positive for the genus *Theileria*, suggesting a low circulation in horses and therefore a very low transmission to ticks. While the risk of introduction of some pathogens belonging to the genus *Theileria* causing tropical theileriosis was questioned with the

arrival of *H. marginatum* in France*, T. annulata* DNA has not been detected in the collected ticks of this study, neither *T. buffeli*, a benign cattle pathogen, previously detected in Corsica (ICTTD, 2000). Curiously, in two third of the cases, we were not able to identify the species of *Theileria* because of unsuccessful sequencing. In these cases, we can make several hypotheses. For ticks collected from cattle, we can wonder if it is *T. annulata* or *T. buffeli* because they are both present in cattle, despite no other species than *T. equi* was characterized. It could also be *T. equi*, as five different genotypes are known in the world and cannot be all amplified using the same primers, or *T. haneyi*, detected since 2018 in USA (Knowles et al. 2018), that may also potentially be considered as an agent of EP (Knowles et al. 2018; Sears et al. 2019). We have finally detected *B. occultans* in cattle ticks from Corsica. Some studies have detected *B. occultans* in *H. rufipes*, *H. marginatum*, *R. turanicus*, and *H. excavatum* ticks (Aktas, Vatansever, et Ozubek 2014; Ros-García et al. 2011; Orkun 2019; Orkun et al. 2020). So far, only *H. rufipes* has been confirmed as a vector of *B. occultans* (Ozubek et al. 2020) and *H. marginatum* is not described as a potential vector of this pathogen. In our study, it can be assumed that ticks engorged on cows carrying this parasite and got infected during their blood feeding. Nevertheless, it cannot be ruled out that an evolutive adaptation could lead to *H. marginatum*-borne transmission of *B. occultans* in southern Europe. This deserves further investigations in *H. marginatum* ticks collected on cattle populations, as recently done in Morocco (Elhachimi et al. 2021).

One virus was detected in this study and it was the WNV in one individual tick, which was collected from a Corsican cow in 2019 and one pool of ticks from a Corsican horse in the south part of the island. Since cattle do not appear to be involved in the WNV natural cycle, it can be assumed that the tick has been infected by the virus during its previous blood meal at immature stage, which probably took place on a bird. Birds, unlike cattle, are involved in the WNV cycle and are even considered as reservoirs of the virus as they are able of re-infecting vectors (usually mosquitoes) (Vaughan, Newman, et Turell 2022), and therefore possibly ticks feeding on them. For the tick pool of 10 males from a horse, considering that horse is an epidemiological dead-end for WNV as it does not produce a sufficient viremia to infect vectors and therefore ticks (David et Abraham 2016), and that adult males engorged much less than females even not at all, the transmission of the virus from the horse to the ticks did probably not occur. This raises the hypothesis that *H. marginatum* would be able at least of transstadial transmission of WNV, which is not a sufficient evidence to confirm its full vector competence. However, the horizontal transmission of WNV to an uninfected vertebrate host by *H. marginatum* at immature and adult stages was experimentally demonstrated in Portugal (Formosinho et Santos-Silva 2006). Because the WNV can cause neurological disease and death in people, our results encourage to intensify monitoring of the WNV in *H. marginatum* and consolidate information on the potential transmission of the virus by this tick species.

With the exception of known or suspected endosymbiont, infection prevalence for endemic pathogens remain very low in the tick *H. marginatum*. Its vector competence can be suspected for *T.equi* and *A. phagocytophilum*. Finally, we did not detect any new exotic pathogens that might have been brought in with *H. marginatum* from its original distribution area. On the basis of all this information, and keeping in mind that this epidemiological situation evolves dynamically may change in the future, we suggest that *H. marginatum* represent good sentinel arthropod for anticipating major health risk for humans or animals in France, and deserves to remain highly monitored due to its ability to spread on long distances via bird migrations and terrestrial animal trade. And this was recently illustrated with CCHFV, previously only detected in countries surrounding France and that we very recently identified in *Hyalomma marginatum* in France (Bernard et al., 2024).

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Data, scripts and codes availability

Data are available online: XXXXDOI of the webpage hosting the data https://doi.org/10.5802/fake.doi
The DOI hyperlink should be active

Conflict of interest disclosure

The authors of this preprint declare that they have no financial conflict of interest with the content of this article. XXXXif some of the authors are recommenders of a PCI, indicate it here. XXXX and XXXX are recommenders for PCI XXXX.

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