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BRIEF REPORT

Development of TaqMan real-time PCR and droplet digital PCR protocols for the detection of *Candidatus* Midichloria mitochondrii and evaluation of exposure among wildlife

Alessia Pucciarelli[1] · Lorena Cardillo[1] · Maurizio Viscardi[1] · Gerardo Picazio[1] · Nicola D'Alessio[2] · Giovanni Sgroi[2] · Antonio Rinaldi[3] · Vincenzo Veneziano[4] · Giovanna Fusco[5] · Claudio de Martinis[1]

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Abstract

Candidatus Midichloria mitochondrii (*Ca.* M. mitochondrii), an endosymbiont intracellular bacterium living in the mitochondria of several tick species, especially ixodid species, has been proposed as a potential marker for monitoring tick-bite exposure. Therefore, the present study aimed to develop two different diagnostic methods, TaqMan-based real-time PCR (rt-PCR) and Droplet Digital PCR (dd-PCR), targeting the 16 S rRNA gene and *gyrB* gene for the detection of *Ca.* M. mitochondrii in different wildlife species from several areas of southern Italy. Both techniques were validated using 10-fold serial dilutions of a sequenced positive control up to reach 10^{-6} final dilution. Among wildlife field samples, both the techniques identified *Ca.* M. mitochondrii DNA, although dd-PCR showed higher sensitivity, being able to detect the target DNA in a higher dilution and in several spleen samples scored negative by rt-PCR. Noteworthy, these molecular methods revealed for the first time the presence of *Ca.* M. mitochondrii DNA in red foxes (*Vulpes vulpes*), Eurasian badgers (*Meles meles*), otters (*Lutra lutra*), porcupines (*Hystrix cristata*), European hares (*Lepus europaeus*), and alpacas (*Vicugna pacos*), suggesting a tick-bite exposure of these animals in the study area.

Keywords TaqMan real-time PCR · Droplet digital PCR · Ticks · Wildlife · *Candidatus* midichloria mitochondrii

Introduction

Candidatus Midichloria mitochondrii (*Ca.* M. mitochondrii) is a member of the Midichloriaceae family, within the order *Rickettsiales*, which is an intramitochondrial symbiont of the ovaries of *Ixodes ricinus*, vertically transmitted to all offspring (Comandatore et al. 2021; Lo et al. 2006).

Although it has been demonstrated as *Ca.* M. mitochondrii is strongly associated with *I. ricinus* ticks (particularly females), this endosymbiont has also been detected in a plethora of different tick species worldwide (e.g., *Amblyomma americanum* from the USA, *Amblyomma ovale* from Brazil, *Hyalomma aegyptium* from Algeria, *Haemaphysalis wellingtoni* from Malaysia, *Rhipicephalus microplus* from Mozambique, *Rhipicephalus sanguineus* and *Hyalomma lusitanicum* from Portugal) (Sgroi et al. 2022a). This aspect may indicate that the transmission of *Ca.* M. mitochondrii by tick-bite other than *I. ricinus* cannot be ruled out. For instance, it has been demonstrated that *Ca.* M. mitochondrii regulates the growth of *Rickettsia parkeri* in its vector

✉ Lorena Cardillo
lorena.cardillo@izsmportici.it

✉ Giovanni Sgroi
giovanni.sgroi@izsmportici.it

1 Unit of exotic and vector-borne diseases, Department of Animal Health, Istituto Zooprofilattico Sperimentale del Mezzogiorno, Via Salute, 2, Portici, Naples 80055, Italy

2 Unit of Wildlife and Regional Wildlife Observatory, Department of Animal Health, Istituto Zooprofilattico Sperimentale del Mezzogiorno, Via Salute, 2, Portici, Naples 80055, Italy

3 U.O.S.D. Genetics Bioinformatics and Biobanking, Istituto Zooprofilattico Sperimentale del Mezzogiorno, Portici, Naples 80055, Italy

4 Unit of Translational Medicine, Department of Veterinary Medicine and Animal Productions, University of Naples Federico II, Naples 80134, Italy

5 Unit of Virology, Department of Animal Health, Istituto Zooprofilattico Sperimentale del Mezzogiorno, Portici, Naples 80055, Italy

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Amblyomma maculatum (Budachetri et al. 2018), turning the light on the antibiotic use towards endosymbionts as a future approach to reduce tick reproductive success (Kolo and Raghavan 2023).

Being horizontally transmitted from ticks to animals and humans *via* blood meals, *Ca.* M. mitochondrii can be serologically and molecularly detected (Mariconti et al. 2012; Bazzocchi et al. 2013; Sgroi et al. 2022a), suggesting the possibility of using this endosymbiont as a marker for monitoring the exposure to tick bites (Mariconti et al. 2012; Sgroi et al. 2022a, b). This application could be useful for monitoring the tick-bite exposure of synanthropic wildlife which, living in human-inhabited areas, may contribute to the circulation of ticks and tick-borne pathogens in suburban and rural areas (Bezerra-Santos et al. 2021). To date, a single quantitative molecular technique is available for *Ca*. M. mitochondrii, based on a relative quantification of the target DNA obtained *via* a SYBR green real-time method targeting a partial fragment of the *gyrase* B subunit gene (*gyrB*) (Sassera et al. 2008; Cafiso et al. 2019). Therefore, this study aimed to develop *(i)* the first TaqMan-based real-time polymerase chain reaction (rt-PCR), and *(ii)* an absolute quantification of *Ca.* M. mitochondrii DNA through Droplet Digital (dd-) PCR among different wildlife species, also comparing the performance of the two diagnostic methods. Thus, two sets of primers and probes were used for the development of both methods: one targeting the 16S s rRNA gene, and the other targeting a partial fragment of the *gyrB* gene (Fig. 1).

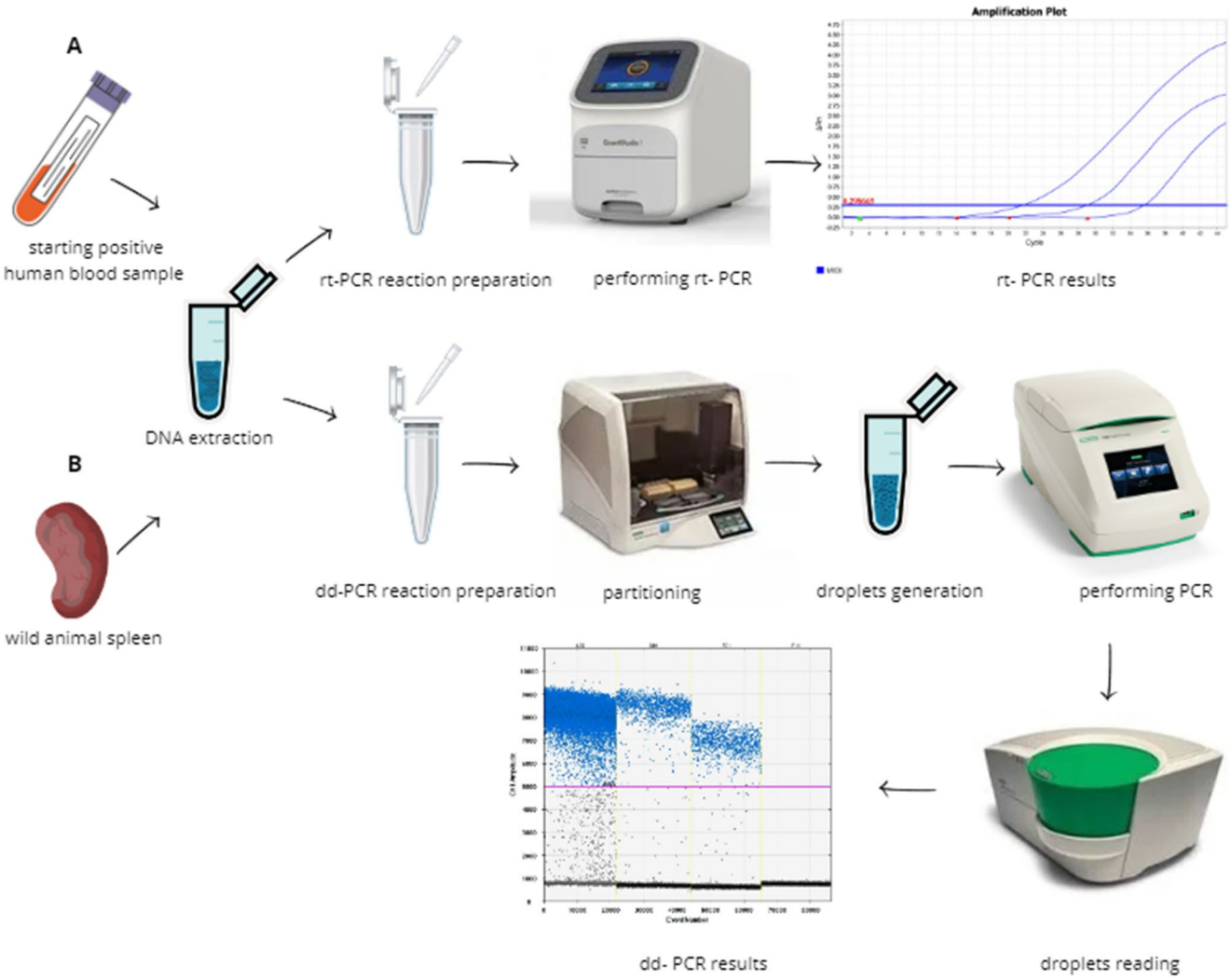

Fig. 1 Molecular investigation workflow. TaqMan real-time and Droplet Digital PCRs. **Stage A)** Starting positive human blood samples for the design of the rt-PCR and dd-PCR. **Stage B)** Wildlife spleen analyses

Materials and methods

Study area and sampling

This study was performed in the frame of a multi-species monitoring plan for zoonoses in wildlife in the Campania region (southern Italy). In this context, 87 carcasses from 13 different species were collected, being 82 wildlife found dead or road-killed and five captive animals dead in zoos and zoological parks in the study area; detailed data are reported in Table 1. The carcasses were conferred to the Wildlife Disease Unit of the Istituto Zooprofilattico Sperimentale del Mezzogiorno (Portici, Southern Italy) for a complete necropsy, also evaluating the presence of any ticks. From each animal, a spleen sample was collected using sterile scalps and stored in sterile plastic tubes at −20 °C.

Nucleic acids extraction, and real-time PCR - droplet digital PCR protocols

Approximatively 2 mg of all spleen samples were processed using the protocol already described by de Martinis and colleagues (2023) for homogenization and nucleic acids extraction. The DNA of the eluates was quantified using the fluorometer Qubit 2.0 (Life Technologies, Invitrogen Division, Darmstadt, Germany). To evaluate the potential inhibition of the samples, the VetMAX Xeno Internal Positive Control (IPC) DNA (Applied Biosystems) was added in the extraction phase, and consequently, a preliminary PCR was performed using the VetMAX Xeno Internal Positive Control (IPC) - VIC (Applied Biosystems), a primer-probe mix that detects the Xeno internal positive control.

Initially, a set of primers was used to develop both rt-PCR and dd-PCR techniques. This primer set (CMM-F-5'-CACA GAAGAAGTCCCGGCTAA-3'; CMM-R-5'-CGCTAGCC CCCTTCGTATTAC-3') and probe (CMM-P-FAM-5'-CGT GCCAGCAGCC-3'-MGB) were designed on a fragment of 61 bp in length, on the basis of a 16S rRNA gene sequence of *Ca.* M. mitochondrii available on GenBank (accession no. MZ954838) (Sgroi et al. 2022b), by using the Primer Expres Software v3.0.1 License (Applied Biosystems).

However, these primers they showed a low grade of sensibility at species level, also in silico. Then, we designed more specific primers for *Ca*. M. mitochondrii, based on the *gyrB* gene. This gene was selected as a powerful molecular tool for identifying and classifying bacteria at the species level, offering greater discriminatory power than the 16S rRNA gene (Wang et al. 2021). The reference genome NC_015722 was used as a template for primer design, which was carried out using Primer3, a widely used open-source software for primer design (Koressaar et al. 2018). To ensure specificity, the primers were then tested in silico using BLAST+ (Camacho et al. 2009) against all available *Ca*. M. mitochondrii genomes deposited in public databases. This approach allowed the selection of primers amplifying specifically *gyrB (*2022a) was used to carry out a 10-fold serial dilution up to reach 10^{-6} final dilution. The dilutions, together with three negative controls, were analyzed in triplicate in rt-PCR and dd-PCR. The same approach was applied using both the primer and probe set targeting the 16S rRNA gene and the *gyrB* gene, respectively. The eluates underwent real–time PCR using QuantStudio 1 PCR thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA) in a total volume of 25 µL, containing 5 µL of template (quantified in a range of 48–51 µg/ml), 12.5 µL TaqMan 2X Universal PCR Master Mix (Thermo Fisher Scientific), 1 µL (0.4 µM final dilution) of each primer and 1 µL of probe (0.2 µM final dilution). The thermal profile was composed of an initial denaturation at 95 °C for 10 min, followed by 45 cycles at 95° C for 10 s and 60 °C for 60 s for annealing and extension, respectively. The two primer and probe sets were used both for rt-PCR and dd-PCR.

Droplet digital PCR was performed using QX200 Droplet Digital PCR Systems (BioRad Laboratories, Hercules, CA, USA) as already described (de Martinis et al. 2023). Samples were considered positive when at least three droplets containing the target DNA were present, while samples were considered as negative when no positive droplet or less than three droplets were revealed, in accordance with the manufacturer's instructions.

Statistical analysis

The Cohen's kappa (k) coefficient was calculated to establish the accordance, repeatability and reliability between rt-PCR and dd-PCR using MedCalc statistical online software (https://www.medcalc.org/calc/kappa.php). The accordance was considered as poor, fair, moderate, strong, and high when showing a k value corresponding to 0–0.4, 0.4–0.6, 0.6–0.8, and 0.8–1, respectively.

Results

All the conferred carcasses were first examined for the presence of any tick. Nevertheless, no ticks were found on the animals examined during necropsy, probably due to the low temperature of the carcasses which easily lead to ectoparasites leaving the body post-mortem.

In the first instance, the performance of the novel rt-PCR and dd-PCR protocols based on the 16S rRNA gene was evaluated for the detection of *Ca*. Midichloria genus. Therefore, starting from the serial dilutions of the positive control a total of 30 samples (7 serial dilutions of the positive control

Table 1 Species of tested animals and results obtained by real-time PCR and droplet digital PCR among positive wildlife samples

		S rRNA gene					*gyrB*				
Species	N. tested animals	N. positive	%	rt-PCR (Ct)	dd-PCR (copies/µL)	dd-PCR (copies/reaction)	N. positive	%	rt-PCR (Ct)	dd-PCR (copies/µL)	dd-PCR (copies/reaction)
Found dead/road-killed											
Red fox (*Vulpes vulpes*)	42	5	11.9	38.7	0.5	2.2	5	11.9	38	0.4	1.7
				38.6	0.5	2.2			37.8	0.4	1.7
				N.D.	0.9	3.9			N.D.	0.6	2.6
				N.D.	2.0	8.8			37.8	0.2	0,8
				N.D.	2.0	8.8			N.D.	4.4	19.3
Eurasian badger (*Meles meles*)	12	1	8.3	37.0	78.0	343.2	-	-	*	*	*
Porcupine (*Hystrix Cristata*)	10	3	30	38.0	1.8	7.9	3	30	36.9	2.3	10.1
				N.D.	0.3	1.3			N.D.	0.2	0.8
				N.D.	0.2	0.9			N.D.	0.2	0.8
European hare (*Lepus europaeus*)	4	1	25	N.D.	0.2	0.8	1	25	N.D.	0.4	1.7
Grey wolf (*Canis lupus*)	4	-	-	-	-	-	-	-	-	-	-
Roe deer (*Capreolus capreolus*)	4	-	-	-	-	-	-	-	-	-	-
Red deer (*Cervus elaphus*)	3	-	-	-	-	-	-	-	-	-	-
Otter (*Lutra lutra*)	3	2	66.6	N.D.	0.2	0.9	2	66.6	N.D.	0.3	1.3
				N.D.	0.6	2.6			38.5	0.5	2.2
Sub total	**82**	**12**	**14.6**				**11**	**13.5**			
In captivity											
Wild boar (*Sus scrofa*)	1	-	-	-	-	-	-	-	-	-	-
Antelope (*Antilope cervicapra*)	1	-	-	-	-	-	-	-	-	-	-
Capybara (*Hydrochoerus hydrochaeris*)	1	-	-	-	-	-	-	-	-	-	-
Alpaca (*Alpaca huacaya*)	1	1	100	N.D.	0.8	3.5	1	100	N.D.	0.2	0.8
Marmoset (*Callithrix jacchus*)	1	-	-	-	-	-	-	-	-	-	-
Sub-Total	**5**	**1**	**20**				**1**	**20**			
Total	**87**	**13**	**14.9**				**12**	**15.1**			

Abbreviations: *Ct,* cycle threshold; *N.D.,* Not detected; *N.A.,* Not applicable

* sample not available

Table 2 Real-time PCR and droplet digital PCR results among 10-fold serial dilutions of the positive control performed in triplicates

Sample	rt-PCR (16S rRNA)		dd-PCR (16S rRNA)			rt-PCR (*GyrB*)		dd-PCR (*GyrB*)		
	Mean (Ct)	SD	Mean (copies/µL)	SD	Copies/reaction	Mean (ct)	SD	Mean (copies/µL)	SD	Copies/reaction
PC	16.2	0.23	saturation	N.A.	saturation	18	0.09	saturation	N.A.	saturation
1:10	18.6	0.28	saturation	N.A.	saturation	22.3	0.09	saturation	N.A.	saturation
1:100	24.5	0.35	saturation	N.A.	saturation	25	0.09	saturation	N.A.	saturation
1:1,000	32	0	4,10E+03	216.02	1,80E+04	28.5	0.35	5,19E+03	66.64	2,29E+04
1:1,0000	35.7	0.32	1,52E+02	15.92	6,67E+02	33	0.09	2,26E+02	14.44	9,94E+02
1:1,00000	39	0.12	15	0.82	6,61E+01	37.5	0.04	1,48E+01	0.74	6,52E+01
1:1,000,000	N.D.	N.A.	1,3	0.12	5,72	N.D.	N.A.	1,3	0.20	5,72
NC	N.D.	N.A.	N.D.	N.A.	0	N.D.	N.A.	N.D.	N.A.	0
NC	N.D.	N.A.	N.D.	N.A.	0	N.D.	N.A.	N.D.	N.A.	0
NC	N.D.	N.A.	N.D.	N.A.	0	N.D.	N.A.	N.D.	N.A.	0

Abbreviations: *Ct,* cycle threshold; *SD,* Standard Deviation; *PC,* positive control; *NC,* negative control; *N.D.,* Not detected; *N.A.,* Not applicable

and 3 negative controls, tested in triplicate) were analyzed in parallel with the two molecular methods. Results are shown in Table 2. The rt-PCR was able to detect *Ca*. Midichloria genus DNA up to 10^{-5} final dilution, while dd-PCR was not able to detect the DNA in the initial positive control and in the first two dilutions (10^{-1}–10^{-2}), as saturation of positive droplets was observed. Furthermore, same results were obtained using the second primer and probe set targeting the *gyrB* gene for the detection of *Ca.* M. mitochondrii species DNA, either by rt-PCR and the dd-PCR (Table 2). Nevertheless, higher sensitivity of dd-PCR compared to rt-PCR was revealed for both the primer and probe sets, as this technique was able to detect the target DNA up to 10^{-6} dilution, both showing an average value of 1.3 copies/µL, corresponding to 5.7 copies/reaction. Furthermore, higher linearity was observed for dd-PCR between the dilutions, which showed a 10-fold reduction in the number of copies/µL, as expected.

After the validation and evaluation of the performance between rt-PCR and dd-PCR, a molecular screening on field spleen samples collected from wildlife was carried out. No sample was found to be inhibited by the evaluation of the IPC. Moreover, there was a difference in the detection of *Ca.* Midichloria genus and species DNA between rt-PCR and dd-PCR. Specifically, using the primer targeting 16S rRNA gene, only 4 out of 87 animals (4.6%) tested positive by rt-PCR. Out of the remaining 83 samples, which had tested negative by rt-PCR, 9 tested positive by dd-PCR, resulting in a total of 13 out of 87 animals (14.9%). For the screening with *gyrB* gene primer set, a total of 86 spleen samples were analyzed, as one spleen from a Eurasian badger was not available for the analysis. In this case, rt-PCR protocol showed that out of 86 spleens samples, 5 (5.8%) tested positive. Out of the remaining 81 samples, which had tested negative by rt-PCR, 7 tested positive by dd-PCR, resulting in a total of 12 (12/86; 13.9%). Furthermore, analyzing both primer sets results, no sample tested positive by rt-PCR and negative by dd-PCR. The results are summarized in Table 1. Finally, when evaluating the performance of the two molecular techniques, the concordance between rt-PCR and dd-PCR was found to be moderate, showing Cohen's $k = 0.45$ for the 16S rRNA gene, while slightly higher concordance was obtained for the *gyrB* gene target showing Cohen's $k = 0.55$.

Discussion

The present study presents the development and application of TaqMan-based real-time PCR (rt-PCR) and droplet digital PCR (dd-PCR) methodologies for the detection of *Ca.* M. mitochondrii DNA in vertebrate hosts. While conventional PCR followed by Sanger sequencing has been previously employed for the identification and characterization of this endosymbiont DNA from both arthropod vectors and vertebrate reservoirs (Epis et al. 2008; Sgroi et al. 2022a), these approaches are inherently labor-intensive, time-consuming, and frequently yield suboptimal results when target DNA is present at low copy numbers. Moreover, conventional PCR lacks the capability of quantification of target sequences. Altogether, these aspects make conventional PCR of limited utility for high-throughput or preliminary screening studies.

In this framework, rt-PCR offers a rapid, and sensitive alternative to conventional PCR. However, its diagnostic performance can be compromised by the presence of PCR inhibitors in complex biological matrices (Schrader et al. 2012; Boulter et al. 2016), and accurate quantification requires the generation of a standard curve for each target (Chen et al. 2021). In contrast, dd-PCR—integrating TaqMan chemistry with droplet-based microfluidic partitioning—addresses these limitations by enabling absolute quantification without the need for standard curves and exhibiting reduced susceptibility to inhibitory substances. This enhanced sensitivity and precision make dd-PCR

particularly advantageous for detecting low-abundance *Ca.* M. mitochondrii DNA in challenging sample types (Chen et al. 2021).

In fact, the dd-PCR protocols herein developed were able to detect the target DNA up to 10^{-6} final dilutions, compared to 10^{-5} positive results of rt-PCRs, and to identify additional positive samples than rt-PCR. Furthermore, dd-PCR is a tallow to obtain absolute quantification, without constructing a standard curve, thereby, quantitative results are obtained, simplifying experimental planning and procedures, while reducing result variability. However, a limit of this technique is identified in the analysis of highly concentrated samples, easily reaching saturation and failing to identify positive samples (Dingle et al. 2013). Moreover, dd-PCR has higher risk of contamination, higher costs, requires longer processing times than rt-PCR and requires higher level of technical expertise as well as personnel training (Campomenosi et al. 2016). Therefore, these aspects clearly indicate that rt-PCR and dd-PCR should be used in combination to avoid missing positive results, or it should be privileged dd-PCR in the case of uncertain samples or in those likely to have low target concentration. When referring to the analyzed gene targets, recent studies have demonstrated that the *gyrB* gene is a powerful molecular tool for identifying and classifying bacteria at the species level, offering greater discriminatory power than the 16S rRNA gene, particularly among closely related strains (Wang et al. 2021). Additionally, the *gyrB* gene has been shown to be an effective phylogenetic marker for exploring bacterial diversity and evolution on relatively short time scales, making it suitable for studies of microbiome evolution and coevolution (Nguyen et al. 2024). Therefore, the use of primers targeting this gene could be more specific for the detection of *Ca*. M. mitochondrii.

Furthermore, this study represents the first report of the presence of *Ca.* M. mitochondrii in foxes, badgers, otters, porcupine, hares, and alpacas, demonstrating the exposure of these animals to tick-bites and consequently demonstrating the risk of exposure to TBPs in rural/peri-urban areas. This hypothesis finds support in a previous study showing that out of 20 human patients tested positive for at least one TBP, 17 (i.e., 85%) were positive also for *Ca*. M. mitochondrii, demonstrating a statistically significant correlation ($p<0.001$, odds ratio = 16.1) between the presence of TBPs and the endosymbiont (Sgroi et al. 2022a, 2022b). Accordingly, from a health perspective, the development of the two molecular methods proposed for the detection of *Ca.* M. mitochondrii could be useful to confirm exposure to tick bites and consequently the probable exposure to TBPs, especially when the presence of the vector on the host is not revealed. Indeed, the absence of ticks on the animals examined during necropsy, ascribable to the low temperatures of the carcasses, further indicates the importance of a screening method for indirectly investigating the presence of ticks on the animals and, consequently, of the potential transmission of pathogens. Considering the strong correlation between *Ca.* M. mitochondrii and *I. ricinus*, it could be of particular interest to further explore the association of *Ca.* M. mitochondrii in other tick species, applying these novel molecular techniques here proposed. Such investigations could provide valuable information to expand the knowledge about the possible wider ecological distribution and potential transmission routes of this endosymbiont.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

Ethics approval In accordance to national/regional regulations and internal policy, the ethical approval was not required being the study based on sample collection performed within routine activities of the health monitoring plan for zoonoses control in wildlife.

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