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Original Article

Molecular Evidence of Spotted Fever Group Rickettsiae in Ticks and Fleas Collected from Some Wild and Domestic Animals in Different Biotopes in Algeria

***Assia Beneldjouzi[1,2], Ismail Lafri[3,4], Amel Djerbouh[3], Safia Nekhili[1], Lydia Hamrioui[1], Nacer Eddine Messahel[5], Zoubir Harrat[1], Naouel Eddaikra[1], Idir Bitam[6,7]**

[1]Laboratoire d'Eco-Epidémiologie Parasitaire et Génétique des Populations, Institut Pasteur d'Algérie. Alger, Algérie
[2]Faculté des Sciences de la Nature et de la Vie, Université de M'hamed Bouguerra, Boumerdes, Algérie
[3]Institut des Sciences Vétérinaires, Université de Blida 1, Blida, Algérie
[4]Laboratoire des Biotechnologies Liées a la Reproduction Animale, Institut des Sciences Vétérinaires. Université Blida 1, Blida, Algérie
[5]Faculté des Sciences Biologiques et Sciences Agronomiques, Université de Tizi-Ouzou, Algérie
[6]umr Vitrome, Aix-Marseille Université, IRD, Service de Santé des Armées, Assistance Publique-Hôpitaux de Marseille, IHU Méditerranée-Infection, 19-21 Boulevard Jean Moulin, Marseille, France
[7]Ecole Supérieure en Sciences de l'Aliment et des Industries Agroalimentaires (ESSAIA), Algiers, Algeria

***Corresponding author:** Dr Assia Beneldjouzi**,** E-mail: a.beneldjouzi@univ-boumerdes.dz

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Abstract

Background: Emergent vector-borne diseases have gained significant attention in recent years due to their increasing prevalence and impact on public health. With its vast geographic and ecological diversity, Algeria has limited available data on the distribution and prevalence of neglected vector-borne diseases. This study aimed to inventory hematophagous ectoparasites, including ticks and fleas, collected from domestic and wild animals such as dogs, hedgehogs, cattle, and rodents across diverse biotopes in northwestern Algeria (Mascara, Oran, Tlemcen, Sidi Bel Abbes, Mostaganem, Tiaret, and Ain Temouchent) and southern Algeria (Laghouat).

Methods: A total of 984 arthropods, comprising 609 ticks and 375 fleas, were collected from domestic and wild animals. Among these, 193 ticks and 105 fleas underwent molecular screening for *Rickettsia* spp. using gltA and ompA gene-specific primers.

Results: The minimum infection rate (MIR) for *Rickettsia* spp. was estimated at 6.37%, assuming one positive individual per pool. Quantitative PCR revealed the presence of *Rickettsia massiliae* in 1/68 (1.47%) of *Rhipicephalus sanguineus* ticks and *Rickettsia felis* in 7/48 (14.58%) of *Ctenocephalides felis* fleas. Additionally, a novel strain of *Rickettsia* sp. was identified in *Rhipicephalus sanguineus* and *Rhipicephalus turanicus*.

Conclusion: This study expands the understanding of tick- and flea-borne *Rickettsia* species in Algeria, highlighting the diverse range of ectoparasite-borne pathogens associated with domestic and wild animals. The findings underscore the importance of continued surveillance and molecular characterization to address the public health risks posed by these pathogens.

Keywords: Algeria; Molecular detection; Spotted fever group rickettsiae; Ectoparasites

Introduction

The obligatory intracellular negative Gram bacteria belonging to the genus *Rickettsia* are the cause of a class of re-emerging infectious illnesses known as rickettsioses (1–2). The primary vectors of rickettsia infection are ticks, fleas, lice, and mites, which can infect people, domestic animals, and wild animals (3–5). These vector-borne zoonotic diseases are distributed

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in all continents and at different latitudes. Many *Rickettsia* species are present on several continents. There are several *Rickettsia* species throughout multiple continents. The vectors of Rickettsiae are linked to their geographic distribution (6–7). In several nations worldwide, rickettsiae represent a major source of illnesses in humans and public health issues (8). They are often separated into two groups: the typhus group and the spotted fever group (SFG). *Orientia* spp. make up the scrub typhus group (9–11).

The *Rickettsia* that causes Rocky Mountain Spotted Fever (RMSF), Mediterranean Spotted Fever (MSF), and several other rickettsiosis cases are included in the purple fever group (3). Mediterranean fever is a spring or summertime bacterial infection mostly seen in the Mediterranean region, and it is caused by *Rickettsia conorii*. *Rhipicephalus sanguineus*, a kind of dog tick, is the carrier of this disease (9, 12–13). Some of the earliest recognized vector illnesses are zoonoses. Since its initial discovery in bacteriology in the late 1930s, *Rickettsia* has drawn more and more attention (14). Currently, the genus *Rickettsia* contains 25 species of harmful bacteria, the majority of which have only been identified in the last 30 years. Ticks belonging to the genus *Ixodes* are the primary carriers of rickettsioses.

Our knowledge of the clinical characteristics and epidemiology of these bacteria has been altered by the discovery of Rickettsiae (15).

But in the last 25 years, the range and significance of rickettsial diseases linked to identified ticks have grown significantly, which makes this disease complex a perfect model for comprehending newly and re-emerging illnesses. New species of *Rickettsia*, whose virulence is yet unclear, are continuously being discovered in or isolated from ticks worldwide. Several vector-borne Rickettsiae, which were previously believed to be non-pathogenic, are now linked to human diseases. This amazing growth of information has been primarily driven by the introduction of molecular biology technologies such as PCR and sequencing, which have facilitated the identification of novel and previously identified Rickettsiae in ticks (16).

Over the past 20 years, there has been a renewed impetus in research on tick-borne rickettsiosis in North Africa, particularly in Algeria, due to a drive from clinicians and entomologists. This is also due to the use of modern tools for the diagnosis and detection of *Rickettsiae* in ticks (17–18).

A current effort from entomologists and clinicians has resulted in twenty years of study on tick-borne rickettsiosis in North Africa, especially in Algeria. This is also because modern methods for diagnosing and identifying Rickettsiae in ticks are being used (17–18). Some vector-borne rickettsial agents, such as *R. aeschlimanii*, *R. massiliae* (11, 19), *R. slovaca*, *R. raoultii*, *R. helvetica*, *R. monacensis* (20–21), and *R. africae* (22) have been found in ticks in Algeria. Flea-associated rickettsioses, including *R. felis*, an emerging SFG rickettsia and *R. typhi*, the agent of murine typhus, are also known to be emerging infections in Algeria (23–24).

Since the disease was placed on the List of Notifiable Diseases (ND) in 1997, the number of cases has continued to increase, reaching a peak in 1999. Subsequently, the number of reports declined during the years 2000–2001, and again a peak was observed in 2002 (25–27).

Monthly Epidemiological Reports (MER) indicate that cases of Mediterranean Spotted Fever (MSF) primarily originated from the Northwest region, particularly four departments: Tlemcen, Oran, Ain Témouchent, and Mostaganem (25–26).

The presence of these microorganisms in these departments has not been extensively researched. Therefore, this study aimed to compile and revise epidemiological information regarding the presence or absence of various infections carried by fleas and ticks, and also to find the risk factors associated with the prevalence of *Rickettsia* spp. in ectoparasites collected from eight departments in northwest Algeria.

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Materials and Methods

Study areas

A collection of ectoparasites was conducted between August 2017 and August 2018 in different biotopes and locations (Fig. 1). In the northwest coastal area, five departments were selected, including Mostaganem, Oran, Ain Temouchent, and Tlemcen. The climate in these regions is humid and semi-humid, with hot summers, cold winters, and an average annual rainfall of 800 mm. In the western interior area, where the climate varies from semi-humid to semi-arid, three departments were chosen. This includes Sidi Bel Abbes, Mascara, and Tiaret. In the southern areas, only one department, Laghouat, was selected, and it has a dry climate (Table 1).

Ectoparasite collection and identification

The investigation was carried out in 17 communities in 8 departments (wilayate) in the northwest of Algeria. The information on specimen collecting is compiled in Table 1.

Beginning in August 2017 and continuing for 13 months straight, the sample was conducted. 198 animal species, both domestic and wild, were inspected for tick and flea infestations during this assessment. To choose animals on which the presence of ectoparasites may be examined, a random sampling technique was employed. The entomological study (Table 1) involved both wild (rodents, hedgehogs) and domestic (cattle, dogs) animals.

Using flashlights, researchers made overnight sweeps around parts of the study sites close to a chicken factory to collect hedgehogs. Acepromazine was used to sedate the hedgehogs, and once the ectoparasites had fully recovered, they were returned to their normal environment. Tiny Folded Aluminum (TFA) Sherman-style traps were used to catch rodents; these traps are designed to catch tiny mammals. Collection of ticks and fleas was done by techniques meant for each type of parasite. Ticks were carefully removed from the skin using forceps, taking care that as much as possible, they should be grasped near the skin to avoid injury to the tick or trauma to the host animal. The ticks were then put in vials with 70% ethanol for preservation, with labels indicating the host species, location, and date of collection. In the case of fleas, fine-toothed flea combs were used to remove them from areas of the animal's body where they are typically found, such as the neck, belly, and tail. Dislodged fleas were collected on a white sheet using forceps and placed into ethanol vials for preservation. A subset of the ectoparasites that were collected was utilized.

All 984 collected ectoparasites were identified morphologically at the species level at the Pasteur Institute of Algeria using standard taxonomic keys (14, 30).

The prevalence of pathogens such as Anaplasmataceae bacteria, *Bartonella* spp., *Borrelia* spp., Piroplasmida, *Rickettsia* spp., and *Coxiella burnetii* in collected ectoparasites was examined using molecular analysis (Fig. 1).

DNA extraction

A total of 298/984 ectoparasite specimens were examined for the presence of the following pathogens: *Coxiella burnetii*, *Borrelia* spp., *Rickettsia* spp., *Bartonella* spp., and *Anaplasma* spp.

Each sample was dried on sterile filter paper discs, longitudinally divided into two equal parts, and washed twice in distilled water for fifteen minutes before DNA extraction. A reserve sample consisting of half of each specimen was kept, minimizing the possibility of sample loss before or after DNA extraction (31). Pools were prepared by grouping ectoparasite specimens from the same species and collection site. Each pool consisted of a predetermined number of specimens (e.g., 5-10 ticks or fleas) to ensure sufficient DNA yield for pathogen detection. The specimens within each pool were thoroughly mixed before extraction to ensure homogenization. DNA ex-

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traction was performed from each pool according to the procedure outlined above.

Following the manufacturer's instructions, DNA extraction was carried out using the Qiamp DNA extraction kit for tissue technique (Qiagen, Hilden, Germany). To reduce the PCR inhibitory impact of the huge amount of blood in the abdomen, the DNA of engorged ticks was extracted from a tiny section of the anterior region of half of the ticks. Using a Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE) and Qiagen AVE buffer as the blank, the total concentration of nucleic acid extracts (2 µL) was measured by absorbance at 260. PCR experiments were performed on DNA samples ranging in size from 50 to 400 ng to detect *Rickettsia* species. Genomic DNA was stored at -20 °C under sterile aseptic conditions.

Pathogens detection

Taqman *Rickettsia* spp., a real-time PCR (qPCR) method unique to the *Rickettsia* genus, was utilized for a screening phase. The gltA gene fragment of these pathogens is the target of the primers and probe utilized (Table 2) (32). Both positive and negative controls were used for every qPCR.

In the Cepheid Smartcycler Automated Real-time PCR System, we ran Taqman real-time PCR tests using 20 µL HotStarTaq Master Mix kit (which includes 250 units HotStarTaq DNA Polymerase, PCR Buffer with 3 mM MgCl2, and 400 µM of each dNTP) and 2×1.7 ml RNase-Free Water. The reaction mixture included 10 µL of Master Mix, 100 nM of forward and reverse primers, 400 nM of dual-labeled probe, 5 µL of template DNA, and up to 20µL of water.

Water was run as a negative control for each test. Cycling conditions included an initial activation of the Taq DNA polymerase at 95 °C for 15 min, followed by 40 cycles: 95 °C for 15 s and 60 °C for 1 min. For every test, water was used as a negative control. For positive control, a sample already known to be positiv e for *Rickettsia felis* and previously confirmed either by PCR or sequencing was used to confirm the validity of the assay.

DNA was extracted from a well-characterized strain of *Rickettsia felis* and was stored at -20 °C until use, after which it was subjected to similar processing conditions to those of the experimental samples. In case of a successful amplification, a band of 787 bp was expected for the positive control. The detection of this positive control in every run of the PCR determined the effectiveness of the reagents and protocol.

The Taq DNA polymerase was first activated at 95 °C for 15 minutes during cycling conditions. This was followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute.

Every DNA sample that showed good results from qPCR was validated by conventional PCR and sequencing for the OmpA gene segments (32). Highly positive samples (Ct <28) were subjected to DNA sequencing procedures. Using *Ri. slovaca*, *Ri. massiliae*, or *Ri. aeschlimannii* qPCR systems, tick samples with Ct >28 were screened by species-specific qPCR by the sequencing results (Table 2) (33).

PCR products were visualized using electrophoresis on a 1.5% agarose gel containing 1X SYBR Safe. *Rickettsia* spp. positive samples' PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) before being forwarded for sequencing.

DNA samples were also analyzed by qPCR for *Anaplasma* spp., *Bartonella* spp., *Borrelia* spp., *Coxiella burnetii*, and *Babesia* spp. (Table 2).

Sequencing

To proceed with Sanger sequencing, the PCR products were purified by filtration and aspiration using NucleoFast 96 PCR plates (Macherey Nagel EURL, Hoerdt, France) following the manufacturer's instructions. A Big Dye sequencing reaction (Big Dye Terminator

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v1.1 Cycle Applied BiosystemTM) was performed on the extracted DNA. Sequencing was carried out with the ABI automatic sequencer (Applied Biosystems).

The analysis of the sequences was carried out using Mega 11 software, and the Basic Local Alignment Search Tool (BLAST) was used to compare the resulting sequences with those found in GenBank.

To create phylogenetic trees, the Mega 11 software was used. A menu with multiple possible models (MrBayes or PhyML) was used to pick the model. The best model is chosen automatically, passed to the menus for starting the phylogenetic analysis, and each model's phylogenetic tree is estimated. Phylogenetic trees were constructed using the Maximum Likelihood (ML) phylogenetic tree that was suggested by the website. Node numbers represent percentages of the bootstrap values that are acquired during 100 cycles of analysis to produce a majority consensus tree (Fig. 7). Citrate synthase (gltA) gene sequence alignments were performed using the following sequences: Alignments were done with the citrate synthase (gltA) gene sequences of: *Rickettsia massiliae* (OQ123707.1, MW978779.1) from Tunisia, France (HM050293.1, U59719.1) and China (MK294042.1); *Rickettsia felis* from China (MK294045.1), Hungary (EU853837.1), Gabon (JQ674484.1); *Rickettsia conorii* subsp. *raoultii* (MN442611.1) from Spain, France (KY073125.1); *Rickettsia aeschlimannii* (OQ 123688.1) from Tunisia; *Rickettsia africae* (MT833658.1) from Algeria; *Rickettsia* sp. From Algeria (JN943294.1), Gabon (OQ 320524.1), and Morocco (OQ633059.1).

Statistical analysis

By dividing the number of afflicted hosts by the total number of hosts investigated, the study's prevalence of ectoparasite infestation was calculated and reported as a percentage.

To examine the variations in the species proportions of ticks and fleas between the research locations, descriptive statistics on the prevalence data were carried out using GraphPad Prism software version 10.0.0 for Windows, GraphPad Software, Boston, Massachusetts, USA (www.graphpad.com).

In addition, a one-way ANOVA was performed to evaluate the mean ectoparasite load between factors using the departments and season as independent variables and the tick and flea species count as the dependent variable. In every statistical test, a p-value of less than 0.05 was deemed statistically significant (34).

Results

Distribution of identified ectoparasites

A total of 120 dogs, 33 rodents, 41 cattle, and 4 hedgehogs were examined for tick and flea infestation, of which 94 dogs, 21 rodents, 27 cattle and 4 hedgehogs were infested, an overall respectively prevalence of 80.83%, 63.63%, 61.36% and 100% (Fig.2).

A total of 984 arthropods were collected from domestic and wild animals. The study resulted in the collection of 609 ticks, including 500 adult ticks and 109 immature ticks (24 nymphs and 85 larvae). 532 were collected from dogs (including 232 ticks and 300 fleas), 165 from rodents (with 90 ticks and 75 fleas), 279 ticks from cattle, and only 8 ticks from hedgehogs (Fig. 2).

Six species of ticks, belonging to three genera, have been identified in our investigation. These were *Rh. turanicus* 38.91% (237/609), *Rh. sanguineus s.l.* 22.66% (138/609), *Rh. bursa* 16.74% (102/609), *Hy. lusitanictum* 13.46% (82/609), *Hy. marginatum* 7.71% (47/609) and *He. punctata* 0.49% (3/609) (Table 3). The current study demonstrated that variations existed in tick variety based on the survey locations (Table 3).

Four species were present in a single department, namely: *Rh. bursa* in Ain Timouchant, *Hy. lusitanicum* in Mostaganem, *Hy. marginatum* in Sidi Belabbes and *H. punctata* in Tiaret (Fig. 3).

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A total of 375 fleas were removed from dogs (300) and rodents (75). The collected fleas were identified morphologically as *X. cheopis* (n= 173; 46.13%), *C. felis* (n= 146; 38.93%), and *C. canis* (n= 56; 14.93%). In dogs, 300 fleas belonging to three species were collected: 146 *C. felis*, representing 38.93% of the Siphonaptera population, 98 *X. cheopis*, forming 26.13 % of the population, and 56 *C. canis*, constituting 14.93% of the settlement. Among rodents, 75 fleas belonging to a single species were collected: *X. cheopis*, 20% of the flea population. The relative abundance of each of these dogs and rodents is presented in Table 4.

During this study, the species *X. cheopis* was collected in 5 departments, namely: Mascara (64), Mostaganem (34), Oran (42), Laghouat (32), and a single flea in Tlemcen (Table 4).

Ctenocephalides felis was found in two geographical zones: Oran (93 specimens) and 53 in Tlemcen. Moreover, 44 *C. canis* were recorded in Laghouat and 12 in Oran (Table 4).

The departments of Ain Timouchant, Tiaret, and Sidi Belabbes have not revealed the presence of fleas.

Out of the examined animals, 109/198 (55.05%) were infested with fleas, 375 of which were found in this investigation. The following data can illustrate the proportions of infested hosts by species: Dogs 88/198 (44.44 %) and rodents 21/198 (10.60%).

Despite this, a non-significant difference in the distribution of ticks and fleas in the eight departments under study was found using the one-way ANOVA. We observed a very significant presence of all species of ticks in the department of Mostaganem, with a predominance of *Rh. turanicus.* However, the three flea species identified during the study (*Xenopsylla cheopis*, *Ctenocephalides felis*, and *Ctenocephalides canis*) were particularly abundant in the Oran department (Fig. 4).

Pathogen detection

DNA was extracted from a total of 298 specimens (193 ticks and 105 fleas). 19 pooled samples containing rickettsia DNA, with a prevalence of infection of 6.37%. In ticks, the overall prevalence was 6.21% (12/193), led by *Rh. turanicus* (MIR 10.34%), followed by *Rh. sanguineus* s.l. with a rate of 4.41% (3/198). Regarding the fleas, 7 out of 105 contained rickettsia DNA (MIR 8.57%). In detail, the partial gltA gene was amplified from *C. felis* only with (MIR 14.58%) (Table 5) (Fig. 5-6). DNA samples were analyzed by qPCR for *Anaplasma* spp., *Bartonella* spp., *Borrelia* spp., *Coxiella burnetii*, and *Babesia* spp. However, no positive were found.

Sequence homology analysis

In the locality of Mascara, only one sample (1.47%) of the species *Rh. sanguineus* s.l. collected from a hedgehog was positive for *R. massiliae* after sequencing of the *gltA* amplicon, showing 100% (661/661) similarity with *R. massiliae* isolate CE83 citrate synthase (*gltA*) gene, partial cds (GenBank Accession no OK314860.1). In Oran, 7/48 (14.56%) of *C. felis* collected from dogs were positive for *R. felis* with an identity of 100% (531/531) to *R. felis* clone CtML-23 citrate synthase (gltA) gene, partial cds (GenBank Accession no OK314861.1). However, a sequence homology analysis of the gltA gene fragment revealed that 11 sequences of the *gltA* gene from *Rh. turanicus* and *Rh. sanguineus s.l.* ticks were identical and showed 100% identity (556/556bp) to *Rickettsia* sp. RDa420 (OK 314862.1) (Table 5).

Phylogeny

Whether dendrograms were created from the gene or the resulting protein, most Rickettsiae had a comparable structure in the dendrograms produced by the tree-building analytic techniques employed.

Two monophyletic groups were spotted in the tree: one consisted of an uncultured *Rickettsia*, *R. conorii* subsp *raoulti* and *R. felis*, and the second of *R. massiliae*, *R. felis*, *R. aeschlimannii*, *R. africae*, and *Rickettsia* sp.

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According to the established phylogeny, we grouped *R. felis* and *Rickettsia* sp. of our specimens with GenBank data of citrate synthase gltA gene sequences from *R. felis* from China (MK294045.1), Hungary (EU853837.1), and Gabon (JQ6744841). *Rickettsia massiliae* patterns were grouped with *R. massiliae* patterns (OQ123707.1, MW978779.1) from Tunisia, France (HM050293.1, U59719.1), and China (MK2940425).

To visualize the phylogenetic relationships, Figure 2 shows a maximum-likelihood phylogenetic tree of Rickettsia based on the partial sequence of the gltA gene (~348 bp), representing the evolutionary relationships of sequences obtained from ticks collected in northwestern Algeria during the years 2017–2018. The tree was constructed using MEGA11 (http://www.megasoftware.net). Based on the Bayesian information criterion (BIC), the Tamura 3-parameter model with gamma-distributed rate heterogeneity and a fraction of invariant sites (T92 + G + I) was selected as the optimal evolutionary model. GenBank accession numbers, species names, and strain identifiers are displayed on branch labels. The sequences highlighted in yellow boxes represent the newly identified Rickettsia sequences generated in this study. Bootstrap values support the reliability of the tree topology.

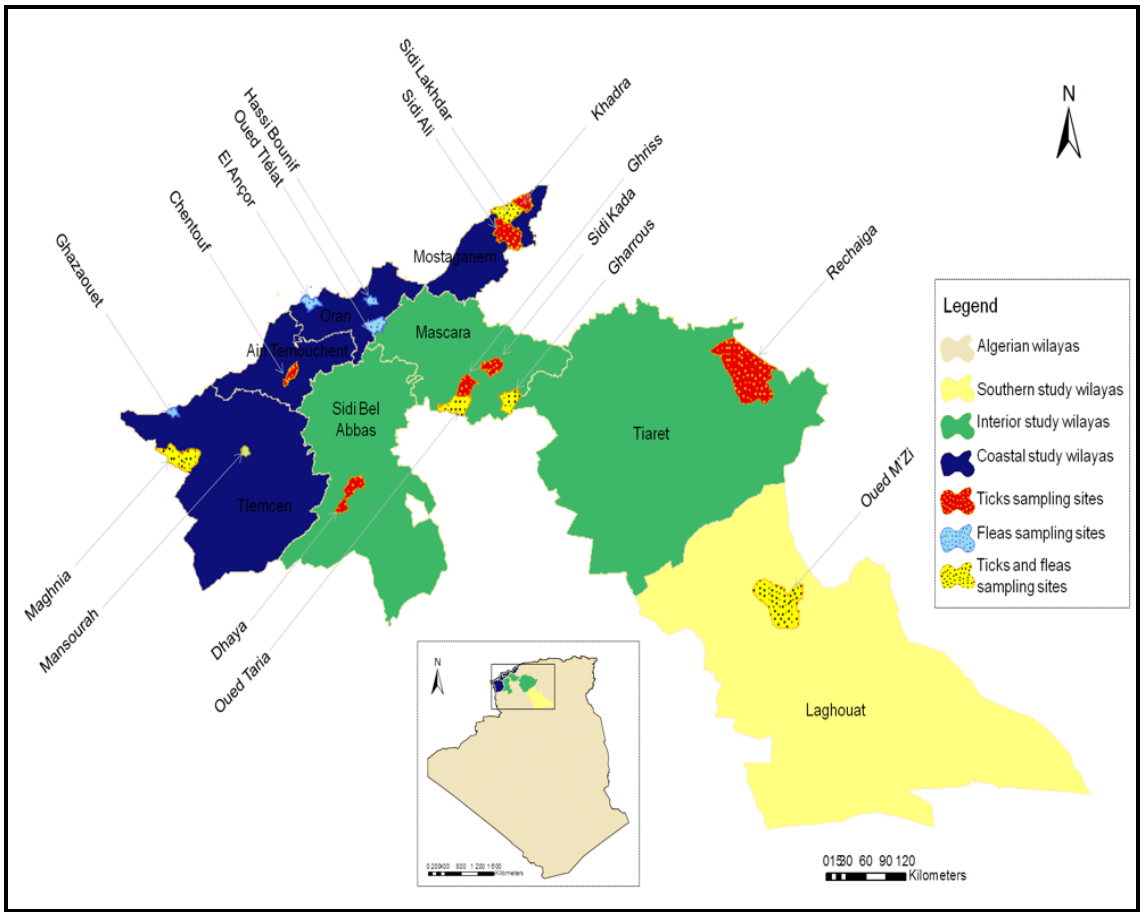


Fig. 1. The locations of the arthropod collecting sites and the research regions in Algeria's northwest and south are indicated geographically. The map was created using ArcGIS 10.3 software

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Table 1. Description of the sites sampled, sources, and stage of arthropod species identified during the entomological survey carried out in Algeria, 2017–2018

Province	Collection site	Coordinates	Source (number of ticks, fleas)	Species	Stage N: nymph L: Larva A: Adult
Mascara	Oued Taria	35° 06′ 53″ N 0° 05′ 19″E	Rodent (9, 39)	*Rhipicephalus turanicus* (9)	N
				Xenopsylla cheopis (39)	A
	Ghriss	35° 14′ 53″N 0° 09′ 41″E	Rodent (1,0)	*Rh. sanguineus s.l.* (1)	A
			Dog (19,0)	*Rh. Sanguineus s.l.* (19)	
			Hedgehog (3,0)	*Rh. turanicus* (3)	
	Sidi Kada	35° 20′ 00″N 0° 21′ 00″ E	Rodent (15, 0)	*Rh. turanicus* (15)	N
			Hedgehog (1,0)	*Rh. sanguineus s.l.* (1)	A
	Gharrous	35° 11′ 45″N 0° 24′ 03″ E	Rodent (0, 25)	*X. cheopis* (25)	A
			Hedgehog (1,0)	*Rh. turanicus* (1)	
	Hassi Bounif	35° 42′ N, 0° 30′W	Dog (0,75)	*X. cheopis* (42)	A
				Ctenocephalides canis (12)	
				C. felis (21)	
Oran	Oued Tlélat	35° 33′ N, 0° 27′W	Dog (0,40)	*C. felis* (40)	
	El Ançor	35° 41′ 12″N, 0° 52′ 08″ W	Dog (0,32)	*C. felis* (32)	
	Sidi Lakhdar	36° 08′ 34″ N 0° 27′ 38″E	Cattle (85,0)	*Hyalomma lusitanicum* (37)	A
				Rh. turanicus (48)	A
			Dog (10,34)	*Rh. turanicus* (10)	A
				X. cheopis (34)	A
Mostaganem	Sidi Ali	36° 06′ 00″ N 0° 25′ 00″ E	Cattle (26,0)	*Hy. Lusitanicum* (26)	A
			Dog (116,0)	*Rh. turanicus* (71)	A
				Rh. sanguineus s.l. (45)	L
	Khadra	36° 15′ 00″ N 0° 35′ 00″ E	Dog (33,0)	*Rh. turanicus* (33)	A
			Cattle (19,0)	*Hy. Lusitanicum* (19)	A
	Mansourah	34° 52′ 16″ N 1° 20′ 21″ W	Rodent (40,0)	*Rh.sanguineus s.l.* (40)	L
Tlemcen	Maghnia	34° 51′ 42″N 1° 43′ 50″ W	Rodent (0,1)	*X. cheopis* (1)	A
			Dog (27,24)	*Rh. sanguineus s.l.* (27)	
				C. felis (24)	
	Ghazaouet	35°5′37″N 1°51′37″W	Dog (0,29)	*C. felis* (29)	A
Sidi Bel Abbes	Dhaya	34° 40′ 31″ N, 0° 37′ 15″ W	Cattle (47,0)	*Hy. Marginatum* (47)	A
			Dog (25,0)	*Rh. turanicus* (25)	
Ain Temouchent	Chentouf	35° 18′ 13″N 1° 01′ 46″ W	Cattle (102,0)	*Rh. bursa* (102)	A
Tiaret	Rechaïga	35° 24′ 29″ N 1° 58′ 24″ E	Hedgehog (3,0)	*Heamaphysalis punctata* (3)	A
Laghouat	Oued M'zi	33° 55′ 27″ N, 2° 26′ 13″ E	Rodent (0,10)	*X. cheopis* (10)	A
			Dog (27,66)	*Rh. Sanguineus s.l.* (05)	
				Rh. turanicus (22)	
				C. canis (44)	
				X. cheopis (22)	

http://jad.tums.ac.ir
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Table 2. Primers, probes, and target sequences used for qPCR detection of bacterial DNA in ticks and fleas

Quantitative real-time PCR designation and specificity	qPCR System used	Forward Primer 5'-3'	Reverse Primer 5'-3'	Probe
Rickettsia felis	Biotin synthase	ATGTTCGGGCTT CCGGTATG	CCGATTCAGC AGGTTCTTCAA	6-FAM-GCTGCGGCGGTATTTT AGGAATGGG-TAMRA
Rickettsia aeschlimannii	Scal	AAGCGGCACTTT AGGTAAAGAAA	CATGCTCTGCA AATGAACCA	6FAM-TGGGGAAATATGCCGT ATACGCAAGC-TAMRA
Rickettsia massiliae	R.mass_9666	CCAACCTTTTGT TGTTGCAC	TTGGATCAGTG TGACGGACT	6FAM-CACGTGCTGCTTATACC AGCAAACA-TAMRA
Rickettsia slovaca	R.slov	GCAACGGTTTTT GGTATCGT	AATCGAATGC ACCACCACTT	6FAM-TCCCGTCCCAGCCATTC GTC -TAMRA
Anaplasma phagocytophilum	Aph-apaG	TAAGCGCAGTTG GAAGATCA	CGGCACATCC ACATAAAACA	6-FAM-TGATGAACGGCTGGTA TCAG TAMRA
***Borrelia* spp.**	TTB23S	CGATACCAGGG AAGTGAAC	ACAACCCYMT AAATGCAACG	6-FAM-TTTGATTTCTTTTCCTC AGGG-TAMRA
Coxiella burnetii	IS1111	CAAGAAACGTAT CGCTGTGGC	CACAGAGCCA CCGTATGAATC	6-FAM-CCGAGTTCGAAACAAT GAGGGCTG-TAMRA
***Bartonella* spp.**	BartoITSr	GGGGCCGTAGCT CAG-CTG	TGAATATATCT TCTCTTCACAA TTTC	6-FAM-CGATCCCGTCCGGCTC CACCA-TAMRA
***Babesia* spp.**	TTBab	GTAGGGAATTGG CCTACCG	TTCTTGTCACT ACCTCCCTGTG	6-FAM-CATCTAAGGAAGGCAG GCGGCGGT-TAMARA

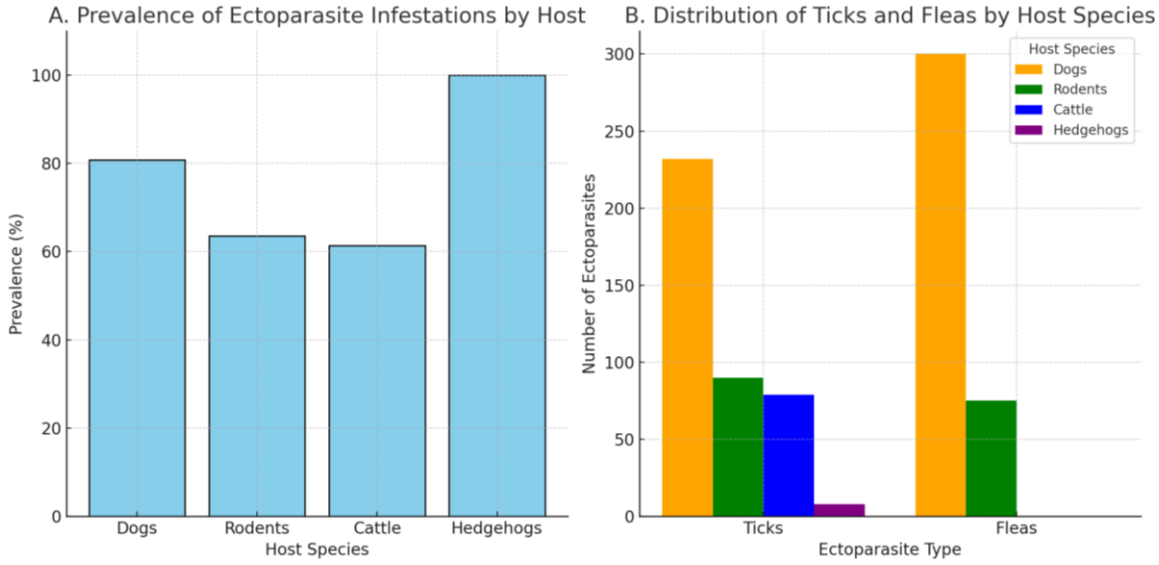


Fig. 2. Prevalence of ectoparasite infestations and distribution of ticks and fleas among different host species in north-western and southern Algeria. A: Percentage of animals infested with ectoparasites by host species. B: Distribution of ticks and fleas collected from each host species

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Table 3. Geographic distribution and abundance of tick species in Northwestern Algeria by department, 2016–2017

Department	*Rh. Sanguineus s.l.*	*Rh. turanicus*	*Rh. bursa*	*Hy. lusitanicum*	*Hy. marginatum*	*H. punctata*	Total
Mascara	21	28	0	0	0	0	**49**
Mostaganem	45	162	0	82	0	0	**289**
Ain Timouchent	0	0	102	0	0	0	**102**
Laghouat	5	22	0	0	0	0	**27**
Oran	0	0	0	0	0	0	
Tlemcen	67	0	0	0	0	0	**67**
Tiaret	0	0	0	0	0	3	**3**
Sidi Belabbes	0	25	0	0	47	0	**72**
Total	**138**	**237**	**102**	**82**	**47**	**3**	**609**

Table 4. Geographic distribution and abundance of flea species by department in Northwestern Algeria, 2016–2017

Department	*X. cheopis*	*C. felis*	*C. canis*	Total
Mascara	64	0	0	**64**
Mostaganem	34	0	0	**34**
Ain Timouchent	0	0	0	**0**
Laghouat	32	0	44	**76**
Oran	42	93	12	**147**
Tlemcen	1	53	0	**54**
Tiaret	0	0	0	**0**
Sidi Belabbes	0	0	0	**0**
Total	**173**	**146**	**56**	**375**

Table 5. Number of positive arthropods and minimum infection rate (MIR) for various tick and flea species carrying *Rickettsia* spp. The data were obtained from samples collected between 2016–2017 in the localities of Mascara, Tlemcen, Mostaganem, Sidi Bel Abbès, and Oran, Algeria. Positive species were identified through targeted gene sequencing and compared to the most similar entries in GenBank

Arthropod species (n)	Positive (n)	MIR (%)	*Rickettsia* spp.	Accession Number of the most similar entry in GenBank	Hosts	Localities
***Rh. sanguineus s.l.* (68)**	3	4.41	*R. massiliae*	OK314860.1	Hedgehog Rodent	Mascara Tlemcen (2016–2017)
***Rh. turanicus* (87)**	9	10.34	*Rickettsia* sp. *Rickettsia* sp.	OK314862.1	Dog Rodent	Mostaganem, Sidi Bel Abbes Mascara (2016–2017)
***Rh. bursa* (07)**	0	0	-	-	-	-
***Hy. marginatum* (10)**	0	0	-	-	-	-
***Hy. lusitanicum* (19)**	0	0	-	-	-	-
***C. felis* (48)**	07	14.58	*Ri. felis*	OK314861.1	Dog	Oran (2016–2017)
***C. canis* (7)**	0	0	-	-	-	-
***X. cheopis* (50)**	0	0	-	-	-	-
Total (298)	19	6.37	-	-	-	-

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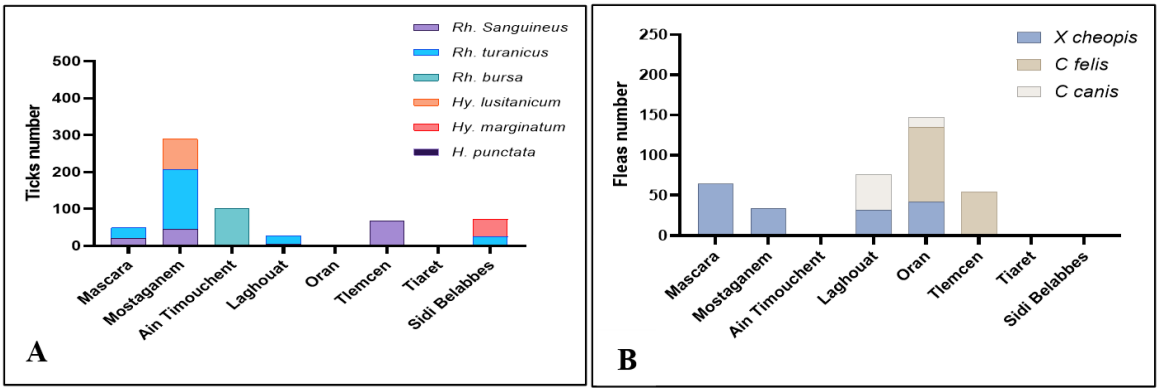


Fig. 3. Total ectoparasite distribution (tick: panel A, and flea: panel B) is seen in the eight ecological zones in Algeria. During August 2016 to August 2017, both domestic and wild animals (cattle, rats, dogs, and hedgehogs) were inspected

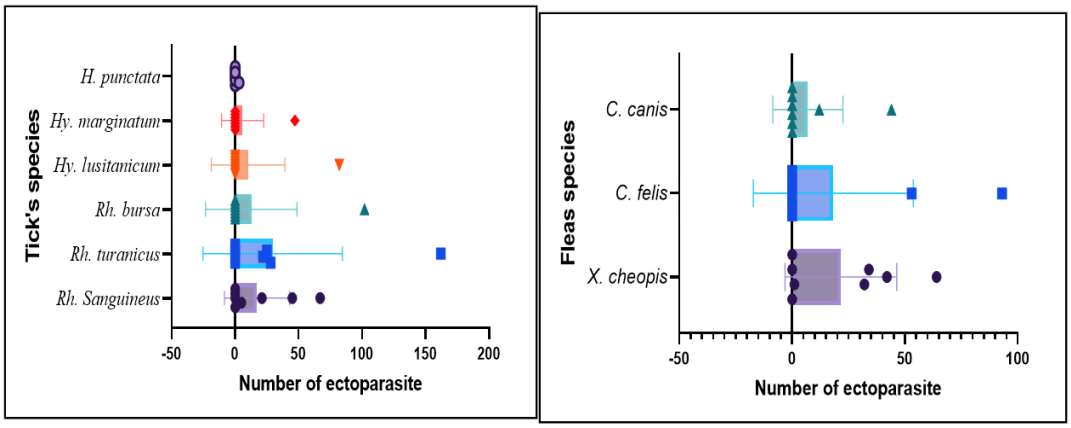


Fig. 4. Distribution of the different ectoparasite species found in the eight research departments that were collected from animals between 2016 and 2017 in Algeria. A: Total number of ticks collected (represented by bars). B: Number of collected fleas (depicted as boxes)



Fig. 5. Agarose gel electrophoresis (1.5%) results showing the amplification of a 787 bp fragment from the gltA gene of *Rickettsia sp.* in 19 pooled DNA samples extracted from a total of 298 specimens (193 ticks and 105 fleas). The figure displays positive samples (lanes 1, 3, 4, 5, 6, 8, 10, 13, 14, 17, 18) and controls. The molecular weight marker (PM Promega 50bp) is used in lane 12 for reference. T- represents the negative control, and T+ represents the positive control

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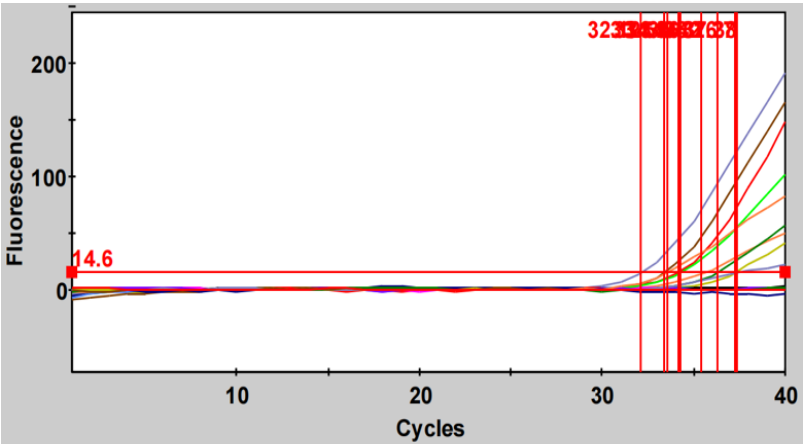


Fig. 6. Fluorescence intensity curves (y-axis) versus cycle number (x-axis) during the PCR amplification using the Smart Cycler Cepheid system. Each curve represents the amplification of pooled DNA extracted from ticks and fleas at different concentrations. The red line denotes the negative control. Positive samples showed fluorescence intensity increasing after cycle 30, indicating successful target amplification

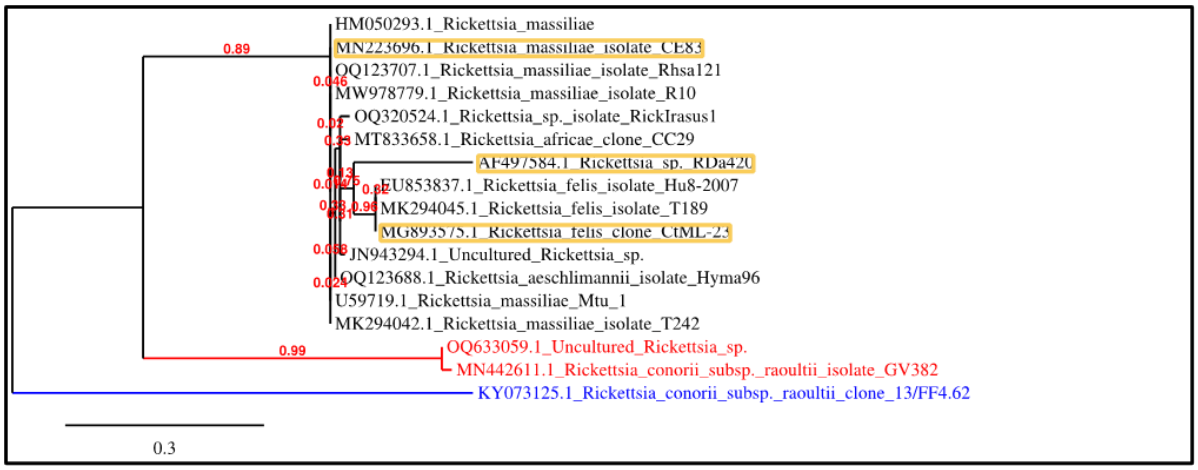


Fig. 7. A maximum-likelihood phylogenetic tree of *Rickettsia* based on the partial sequence of the gltA (~348 bp), showing the evolutionary relationships of sequences obtained from ticks collected in northwestern Algeria during the years 2017–2018. The tree was constructed using MEGA11 (http://www.megasoftware.net). Based on the Bayesian information criterion (BIC), the Tamura 3-parameter model with gamma-distributed rate heterogeneity and a fraction of invariant sites (T92 + G + I) was selected as the optimal evolutionary model. GenBank accession numbers, species names, and strain identifiers are displayed on branch labels. The sequences highlighted in yellow boxes represent the newly identified *Rickettsia* sequences generated in this study. Bootstrap values support the reliability of the tree topology

Discussion

Ectoparasites are a large group of parasitic arthropods that fall into two distinct classes: Insects, which include the order Siphonaptera, and Arachnids, which include the class Ixodidae. The latter are in charge of spreading various pathogens that can result in vector-borne illnesses and zoonoses.

In this work, we documented the identification of spotted fever group Rickettsiae DNA in ectoparasites obtained from both domestic and wild animals in various Algerian biotopes and regions. Ticks do not have parasitic specificity towards their hosts. We are talking about trophic preferences; the same host can be par-

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asitized by different species of ticks, the case in this study.

The existence of appropriate climatic conditions and host adaptation can be associated with the occurrence of these arthropods in the locations that we investigated. A total of six tick species were obtained, including:

The three-host tick, *Rh. sanguineus* is primarily obtained from dogs, which is also its preferred host (35). Only a limited number of individuals have been discovered in small animals. Because it is commonly associated with dogs, which are thought to be the primary reservoir of *R. conorii*, this tick is well suited to both urban and rural human habitats. In this study, 96 out of 138 *Rh. sanguineus* s.l. were collected from dogs. This tick is often found on dogs, but it is also occasionally seen on people and a variety of domestic and wild animals, such as cats, rats, and birds. These results are in perfect match with those obtained by Dantas-Torres et al. (36) and Hornok et al. (37). Nonetheless, there are very few reports of *Rh. sanguineus* in hedgehogs (38).

In the present study, *Hy. marginatum* was collected from March to November in open biotopes (meadows and areas of domestic crops) only on cattle. These constitute the principal host of this species; however, it can infest other animals such as camels. Djerbouh et al. reported the presence of this tick species in southern Algeria on camels (39).

Hyalomma lusitanicum had an annual activity (40–41); It is the species that has the highest parasite load on cattle. It was mainly harvested in the wooded and bushy areas of the region of our study. *Rhipicephalus turanicus* had a relatively short seasonal activity, extending from April to August (37–42). In this study, the distribution of this species was much larger than that of *Rh. bursa*. However, these results agree with those found by Boulkaboul in 2003 in the region of Tiaret, where *Rh. bursa* was collected from cattle (43).

There were no species of ticks found in the Oran department, which is in contrast to the findings of Yousfi-Monod and Aeschlimann, who collected 5500 ticks from bovids in the western region of Algeria (44). This is explained by the fact that only dog samples were collected and by the area's urbanization, which has prevented any breeding.

The ixodid fauna of Algeria is not well understood in terms of its unique biology or biodiversity. The still-old literature on this topic needed to be revised (14).

The main vectors of *R. massiliae* transmission are ticks belonging to the *Rhipicephalus* genus. The discovery of *R. massiliae* in *Rh. sanguineus* ticks taken from dogs, hedgehogs, and small rodents supported this pathogen-vector relationship and highlighted the potential link between *R. massiliae* and human rickettsiosis in the northwest of Algeria. Only three cases of human rickettsiosis caused by *R. massiliae* have been reported and verified by genetic methods in Europe; two of these cases happened in Sicily (45–47). The initial case was found in a blood sample taken from a patient from Sicily who was admitted to the hospital due to a skin rash and fever (46). The patient in the second case had spotted fever and a sudden decrease in vision; he was found in southern France (47). In the third instance, a young patient from Sicily showed scalp eschar and neck lymphadenopathy (45).

In Algeria, *R. massiliae* was detected for the first time in 4 specimens of *Rh. turanicus* (2 collected from cattle, 1 collected from goats, 1 collected from a hedgehog) and 4 specimens of *Rh. sanguineus* (collected from a hedgehog) (19). *Rickettsia massiliae* was recently found in *Rh. bursa* and *Rh. sanguineus* s.l., indicating that it is present throughout the country (48). It is uncertain if *R. massiliae* is harmful to humans. On the other hand, the fact that it was found in tick saliva raises the possibility that bacteria could be transmitted by a tick bite. For all these reasons, additional research is needed for the identification of *R. massiliae* in Algerian ticks and patients.

Moreover, the cat flea species *C. felis* is known

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for carrying the spotted fever agent *R. felis*, an emerging pathogen that causes infectious diseases in people (49–50). Since then, *R. felis* infections have been discovered in 12 species of fleas, 8 species of ticks, and 3 species of mites (50). *Aedes albopictus* mosquitoes in Sub-Saharan Africa have also lately been found to have Rickettsiae (23, 51).

In our study, we also reported *R. felis* infection in *C. felis* collected from dogs in two localities considered endemic to rickettsioses in Algeria, Oran and Tlemcen. These results are per the studies of Bitam et al. (23) and Mouffok et al. (52). The values of the minimum infection rates (MIR) reported by our investigation results for *C. felis* are similar to those previously reported in some provinces of Brazil (14.3%) and the United States (13.3%) (53–54). However, they are higher than the MIRs reported in other countries, such as Colombia (5.3% MIR) (55) and Taiwan (8.2%) (56). Several studies have highlighted the wide distribution of *R. felis* infection in *C. felis*, with varying proportions of infection. For instance, in Mexico, 20% of the 54 *C. felis* pools collected from dogs were infected (57). In addition, 64% (55/86) and 58% (47/81) of pools of *C. felis* collected from cats and dogs were found to be positive for *R. felis* infection in Guatemala and Costa Rica, respectively (58); while 41% of the pools taken from 15 cats and dogs (25/62 *C. felis* and 2/4 *C. canis*) were reported infected in Uruguay (59). In Algeria, *R. felis* was first detected in *Archeopsylla erinacei* fleas collected from hedgehogs in the Oran district in 2003 by Bitam et al. (23). Besides, other studies have also confirmed the prevalence of *R. felis* in Algeria (24). Recently, DNA of *R. felis* was detected in cat fleas by Bessas et al. (60). In 2010, Kernif et al. (61) introduced a new flea species (*X. cheopis*) as a potential vector of this bacterium in Algeria.

Conclusion

In recent years, the dangers associated with arthropod bites and vector-borne infections have been revealed. It should be remembered that the identification of an ectoparasitic pathogen does not necessarily mean that treatment is required or that the disease has spread to a susceptible host. Health professionals can be alerted by molecular surveillance, which consists of identifying and characterizing pathogens present in collected ticks and fleas, to prevent exposure to certain infections, provide information on potential health risks, and accelerate the process of diagnosis and treatment. The analysis of the sequences of the PCR products and the PCR tests used in this study allowed us to obtain additional information on the epidemiology of tick- and flea-related bacteria in the North-West of Algeria, where there is little information on the subject. This work identified bacteria that are closely related to animal or human pathogens, as well as bacteria whose pathogenicity is unknown. However, DNA detection does not mean that the vector species concerned are capable of transmission, as they could have been collected from bacteremic animals. In addition, due to the limited study time and seasonal fluctuations in arthropod activity, other tick species could be dominant in the other part of the year. In our research, we examined two tick species and one flea species were identified as spotted group Rickettsia in our epidemiological study. It is recommended to carry out more genetic studies on various genes to determine the exact situation of *Rickettsia* species spread by arthropod vectors in Algeria. In summary, the discovery of *Rickettsia* SFG in ectoparasites of domestic and wild animals highlights the importance of vector-borne disease surveillance, particularly in high-risk regions of northwestern Algeria.

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Ethical considerations

Before the collection of ectoparasites, the owners of all domestic animals provided their informed consent. The national legislation (Le Journal officiel n° 47 du 19 Juillet 2006, http://www.iucnredlist.org/ apps/redlist/details/27926/0) and the local ethics committee approved the study on hedgehogs.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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