

Original Article

Serological and Molecular Detection of *Rickettsia* spp in Patients with Meningeal Syndrome in Northern Algeria

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Abstract. Background: Rickettsioses are considered emerging or re-emerging hematophagous arthropod-borne zoonosis. In addition, Meningeal syndromes are among the most common reasons for consultation in infectious disease emergencies.

Objectives: Our study aimed to identify *Rickettsia* spp using IFA and qPCR for serological and molecular tests, respectively, in patients presenting with Meningeal Syndrome at the National Centre of Infectious Diseases EL-HADI FLICI Hospital, Nicolle-Laveran department in Algiers.

Methods: We collected 55 whole blood and 55 sera from patients with meningeal syndrome of various ages and genders, with a mean age of 24.03 (ranging from 2 to 50 years old).

Results: The indirect immunofluorescence assay (IFA) for *Rickettsia* spp returned positive results in seven sera (7/55, 12.72%). We diagnosed four cases of Spotted Mediterranean Fever (MSF) caused by *R. conorii*, two cases of Murine Typhus caused by *R. typhi*, and one case of Flea-borne Spotted Fever caused by *R. felis*. Concerning the spinal tap, we reported that three patients were positive for *R. conorii*, where cerebrospinal fluid was opalescent, and there was a mixed cellular profile on cytology. These findings suggest a possible co-infection with *R. conorii* in patients with meningeal syndrome. Thus, the serological findings coupled with the Meningeal syndrome and the diverse clinical manifestations of both pathogens observed among these patients suggest a possible overlap in clinical expression.

Conclusion: These results highlight the importance of heightened awareness among infectious disease specialists, particularly when faced with confirmed cases of Meningeal Syndrome that may exhibit clinical similarities with Rickettsial diseases, especially in regions where hematophagous arthropod-borne zoonoses are prevalent.

Keywords: Meningeal syndrome, *Rickettsia* spp, Zoonosis, Haematophagous Vector-borne, Algeria.

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Introduction. Rickettsioses are considered as emerging or re-emerging arthropod-borne zoonosis since cases of human infection are continually described worldwide and due to wide geographic distribution to the ecology of their vectors.^{1,2} The causative agent of Rickettsial is an intracellular bacterium from the genus *Rickettsia*, which is phylogenetically classified into four groups: Spotted Fever Group (SFG), Typhus Group (TG), Transitional group and ancestral group.³ Serologically, the SFG rickettsia are similar to the transitional and ancestral groups, but all are distinguishable from the TG rickettsia. Within the SFG, *R. conorii* is the etiological agent of Mediterranean Spotted Fever (MSF), and *R. Rickettsia* is the etiological agent of the Rocky Mountain Spotted Fever. Among the TG, *R. typhi* is the causative agent of Murine Typhus (endemic typhus).⁴ Based on its high sensitivity and specificity, the Immunofluorescence Assay (IFA) is the Gold Standard for serological diagnosis of Rickettsial infections.⁵ The Rickettsioses are transmitted by various arthropod vectors, such as ticks, fleas, body lice and mites.⁶ The host animals of these hematophagous vectors are also important as they transport vectors to the environment in and around places where humans live. When the Rickettsial host-vectors animals are autochthonous, a positive serology is related directly to the circulation of the bacteria in this region.⁷ Different rickettsial infections tend to cause similar symptoms: Fever, severe headache, a characteristic rash (Maculopapular, Purpuric), and a general feeling of illness (malaise). The Mediterranean Spotted Fever is the most common rickettsioses occurring in the Mediterranean region, this disease is transmitted by the bites of the brown dog ticks; *Rhipicephalus sanguineus*.⁸ Algeria is a Mediterranean basin country where the warm climate covers a significant part of the continent. Consequently, several human-vector Rickettsioses have been studied, namely *R. conorii conorii* and *R. massiliae* detected in hematophagous vectors as *Rhipicephalus sanguineus*, *R. aeschlimannii* detected in *Hyalomma marginatum marginatum*;^{2,8} Also 63 sera-human cases came back positive for *Rickettsia conorii conorii* in Western Algeria and other 14 human cases were diagnosed in eastern Algeria.^{6,8}

Meningeal syndromes are also among the most common reasons for consultation in infectious disease emergency departments in Algeria. Its detection requires emergency hospitalisation for a lumbar puncture and cerebrospinal fluid (CSF) analysis to establish an etiological diagnosis. The positive diagnosis of meningeal syndrome is based on the classic triad of

symptoms: headache, vomiting, and neck stiffness. In fact, lumbar puncture remains the gold standard for diagnosing acute meningeal syndrome.⁹

In Algeria today, it is essential to consider the balance between the broad spectrum of Rickettsial symptoms and the limited availability of biological diagnostic tools. Consequently, infectious disease specialists often rely primarily on clinical diagnosis, which creates a significant challenge and directly impacts the therapeutic approach. In light of these considerations, our study aims to highlight the correlation between clinical suspicion of rickettsioses and its detection in patients exhibiting confirmed meningeal syndrome, particularly given the overlapping clinical signs, such as fever and headaches.

Material & Methods.

Study design. To provide evidence, we investigated the relationship between clinical suspicion of Rickettsioses and its confirmation in patients with meningeal syndrome; we considered it wise to focus our study at the National Reference Centre for Infectious Diseases in Algeria, named ELHADI FLICI Hospital, Nicolle-Laveran department, Algiers, which admits patients from all the Algerian departments. Our study was conducted between July and September 2019, which coincides with the summer season, when vector-borne and tropical diseases are most prevalent. In total, 55 patients from various districts of Algeria were admitted to our hospital and included in the study.

Inclusion criteria. To select the patients most suitable for our study, we included only those diagnosed with a Viral Meningeal Syndrome, confirmed clinically with physical meningeal syndrome: Stiff neck and signs of Kernig “Knees extension” and Brudzinski “full flexion of the hips”, and supported by positive findings from a Cerebrospinal Fluid (CSF) analysis: Moderate increase of the White Blood Cell count (50-1000 cells/ μ L), predominantly lymphocytes, and normal protein or slightly elevated (50-200 mg/dL), this step was realised at the central laboratory of ELHADI FLICI Hospital-Algiers in order to confirm the meningeal syndrome. These patients also needed to exhibit at least three of the following clinical signs indicative of Rickettsial infections: sudden onset, high fever, severe headache, arthralgia, myalgia, tick bites, characteristic rash (maculopapular or purpuric), Black Spot, and a general feeling of illness. A total of 55 patients were enrolled in the study: 14 adult males (25.45%), 22 adult females

(40.00%), and 19 children (under 15 years old, 34.54%: 11 males and 9 females); the mean age was 24.03 (2 to 50 years old). In parallel, we completed a questionnaire for each patient to gather additional information, such as epidemiological data, housing area (rural or urban), contact with ticks or animals, profession, and any history of tick bites.

Sample's collection. A total of 55 whole blood and 55 sera samples belonging to positive Meningeal Syndrome patients were collected aseptically in suitable EDTA and dry tubes, respectively. The samples were conserved at -20°C to be handled at the URMITE (Emerging Tropical Infectious Diseases Research Unit at the Institut Hospitalo-Universitaire (IHU) Marseille) for IFA serology and qPCR for the rickettsial spp.

Serological assays. Serologic tests were performed using an indirect immunofluorescent antibody (IFA) assay, which is the reference method for the serodiagnosis of Rickettsioses. At the serological service of the IHU Marseille, we test a panel of Rickettsial antigens, such as *R. conorii*, *R. felis*, and *R. typhi*. Immunoglobulin G (IgG) antibody titers of 1:128, seroconversion in paired serum specimens, with/not IgM antibody titers of 1:32 against any species, were considered evidence of recent *Rickettsia* infection.²

DNA extraction and real-time PCR. A total of 200 µL of DNA was extracted from whole blood tubes using the QIAamp Tissue Kit by QIAGEN-BioRobot EZ1, according to the manufacturer's instructions (Qiagen, Hilden, Germany). Extracted DNA was stored at -20°C under sterile conditions until it was used in PCR assays. Extracted DNA was used in qPCR amplifications of the *gltA* gene of *Rickettsia* species.⁹ Positive samples are further amplified by PCR targeting the outer membrane protein (*ompB*)¹⁰ and citrate synthase (*gltA*) genes.¹¹ The final qPCR reaction mixture consisted of 5 µL DNA with 15 µL of mix from the Roche PCR Kit (Roche Diagnostics, Meylan, France). The PCR cycling parameters for the qPCR were 5 min at 95°C followed by 39 cycles, each consisting of 5 sec of denaturation at 95°C and 30 sec of annealing at 60°C.

Results

IFA Serology. A total of 55 sera were collected from 55 patients.

Table 1 summarises the distribution of signs and symptoms among all patients included in the study. The results shown in **Table 1** lead us to point out that Fever and Arthritis were the most common symptoms for all the patients included in the study (58.18% and 80.00%, respectively). However, only 14.54% of the population study have Black Spots, and 16.36% have a purpuric rash. A maculopapular rash is the most

Table 1. Distribution of Symptoms by Percentage for all Patients in the Study.

Symptoms	Number	Percentage
Fever	32	58.18%
Headache	27	49.09%
Arthralgia	44	80.00%
Myalgia	21	38.18%
Maculopapular Rash	17	30.90%
Purpuric Rash	09	16.36%
Black Spot	08	14.54%

prominent cutaneous symptom of Rickettsial disease; however, we found it in only 30.90% of patients. This variation in symptoms from one patient to another makes a definitive diagnosis challenging.

The screening for all the sera for the antigens against *R. conorii*, *R. felis* and *R. typhi* came back positive for only 07 sera (07/55, 12.72%).

Table 2 reports the IgG and IgM titers against *R. conorii*, *R. felis*, and *R. typhi*, as well as the qPCR results and other signs belonging to positive IFA.

Using IFA against *Rickettsia* spp antigens, we obtained only 07 positive samples from 55 ones (12.72%). Among these positive samples, we note that 04 of them are Female, and the 03 left are male. However, the Dog/Tick contact did not reflect the real infectious status because even with no Dog/Tick contact, we found positive rickettsial titers. Otherwise, according to these IFA results, we diagnosed 04 cases of Spotted Mediterranean Fever (MSF) caused by *R. conorii*, 02 cases of Murine Typhus caused by *R. typhi*, and only one (01) case of Flea-borne Spotted Fever caused by *R. felis*. In parallel, the IFA of patient number 06 came back positive for two (02) Rickettsioses, *R. conorii* and *R. felis*. These cross-reactive antibodies have been confirmed by the Western-Blot test, which makes the result more sensitive in support of *R. conorii* positivity.

The four cases of MSF and one case of Flea-borne Spotted Fever belong to the SFG of Rickettsial diseases; however, the two cases of Murine Typhus belong to the TG of Rickettsioses.

Regarding the positive results obtained and the variation in symptoms among these patients, as shown in **Figure 1**.

In this analysis, we examine the results based on symptoms and the bacterial species involved. Fever and myalgia are consistently observed in all positive patients, regardless of the bacterial species, indicating that these are common symptoms of Rickettsial infections. Additionally, a maculopapular rash is present in half of the patients with both species of *R. conorii* and *R. felis* but is absent in those infected with *R. Typhi*. Nevertheless, purpuric rash is observed in 50% of cases with *R. conorii* but is missing in patients with other strains of *R. conorii* and *R. Typhi*. The feature black spot

Table 2. History of dogs/Ticks contact and IgG/IgM titers against *R. conorii*, *R. felis*, *R. Typhi*, and also the qPCR results in patients with IFA positive samples.

Patient	Sex/age	Contact with dogs/ Ticks	Titers against <i>R. conorii</i>		Titers against <i>R. felis</i>		Titers against <i>R. typhi</i>		qPCR <i>gltA</i> gene
			IgG	IgM	IgG	IgM	IgG	IgM	
01	F/08	YES	n	n	n	n	1:64	1:256	n
02	F/11	NO	1:64	1:32	n	n	n	n	n
03	F/44	YES	1:256	1:32	n	n	n	n	n
04	M/48	NO	n	n	1:128	1:64	n	n	n
05	M/50	YES	1:64	1:32	n	n	n	n	n
06	F/19	YES	1:4096	1:128	1:4096	1:64	n	n	n
07	M/47	NO	n	n	n	n	1:32	1:64	n

*n: negative. *All sera are screened as first-line with Total Immunoglobulin. If the serum is positive at 1/100 dilution, then the antibodies present in this sample are differentially quantified (IgG, IgM).

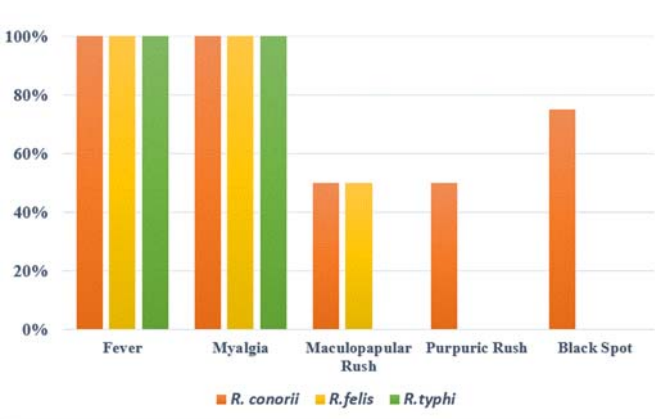


Figure 1. Variation of symptoms across different Rickettsial species.

is found exclusively in cases of *R. conorii*. By recapping, all Rickettsia-positive patients exhibit fever and myalgia, while skin lesions are observed only in patients positive for *R. conorii* and *R. felis*. However, the Tache noire sign is present solely in patients positive for *R. conorii*.

This distribution of symptoms suggests that while fever and myalgia are general indicators of Rickettsial infections, the presence of specific symptoms such as maculopapular rash, purpuric rash, and tache noire might help in differentiating between various Rickettsial species and strains. This information is essential for accurate diagnosis and appropriate treatment.

Detection of Rickettsia spp by quantitative qPCR. qPCR was used to detect *Rickettsia* spp in whole blood by employing *Rickettsia*-specific primers and a probe designed to amplify the *gltA* gene. All the whole blood qPCR came back negative for the Rickettsial gene *gltA*, even in patients whose sera tested positive for any Rickettsial species using IFA. These results could be explained by the samples not coinciding with the Rickettsiae bacteraemia phase.

On the other hand, the Complete Blood Count (CBC) of all the positive rickettsial cases was represented by hyperleukocytosis. Also, for all of them, no thrombocytopenia was observed, and C-reactive protein

(CRP) was normal. Concerning the Spinal Tap, the Cerebrospinal Fluid aspect was clear in 04 rickettsial cases (01 *R.conorii*, 02 *R.felis* and 01 *R.typhi*). However, 03 *R.conorii* showed an opalescent Cerebrospinal Fluid aspect. In the cytology test, the mean was 737 elements/mm³ for the rickettsial cases, with 03 lymphocytic cases (02 *R.felis* and 01 *R.typhi*) and 04 mixed cellular cases (04 *R.conorii*). In addition, we had a slight elevation of Cerebrospinal Fluid Proteins and normal glycorrachia.

Discussion. Aiming to showcase the relationship between clinical suspicion of Rickettsioses in patients with confirmed meningeal syndrome, we focused our study at the National Centre of Infectious Diseases EL-HADI FLICI Hospital in Algiers. During the study period, from July to October 2019, high temperatures were recorded in Algiers (till 41.5 °C); knowing that Algeria is a warm country, vector-borne infectious diseases are widespread in the summer season. It is well known that the distribution of vectors and associated pathogen transmission rates can be affected by changes in ambient temperature and climate.¹ Therefore, other species of ticks are getting into the Rickettsial cycle. Thus, the infected blood of these vectors potentially increases the risks of human infection.

Mariusz Piotrowski and colleagues (2020) reported that the discovery of new rickettsiae has altered our understanding of the clinical features and epidemiology of these bacteria. In addition to the classic symptomatic triad of fever, rash, and headache, it has been demonstrated that each rickettsiosis possesses distinct characteristics, including severity and inoculation rate.¹²

Moreover, the meningeal syndrome is a set of symptoms associated with the pathological irritation of the meningeal envelopes of the central nervous system; this disease was confirmed clinically and biologically by all patients included in our study. Furthermore, a panel of clinical symptoms in favour of Rickettsial infections was required for the inclusion criteria, such as fever,

severe headache, a characteristic rash (maculopapular, purpuric), and a general feeling of illness (malaise). A total of 55 sera belonging to 55 patients were collected aseptically and tested by IFA Rickettsial serology of *Rickettsia* spp. Rickettsial IFA serology came back positive for four of seven sera from the 55 tested (12.72%). The positivity of Rickettsial IFA varies from patient to patient, with precisely 04 cases of Spotted Mediterranean Fever (MSF), 02 cases of Murine Typhus, and only one (01) case of the Flea-borne Spotted Fever. The positive patient's sera for Rickettsioses and the confirmed meningeal syndromes in all these cases, on the one hand, and the various clinical manifestations of Rickettsial and meningeal symptoms among these patients, on the other hand, lead us to suggest the competitive pathogenicity expression between the two pathogens. All positive cases exhibit symptoms of fever or more clinical findings. The most common finding was the presence of Maculopapular Rash and Myalgia. This different expression of clinical symptoms for both pathogens (Meningeal and Rickettsial) depends on the severity of the disease and may range from mild to more severe. It is noteworthy to assess these results concerning the co-infections between the Meningeal pathogen and the *Rickettsia* spp. Moreover, the absence of some key clinical features (for MSF diagnosis) in our patients confirms the clinical polymorphism. It makes the diagnosis difficult, which is supposed to be clinical, leaving the morbidity underestimated in endemic areas. In other words, negative inflammatory assessment is a characteristic of intracellular bacteria. Confirmation is useful for seroprevalence studies but remains late, giving a retrospective diagnosis of interest for knowledge of the clinical description of rickettsioses in general (particularly *R. felis*) and without therapeutic support. This late diagnosis, which results in late treatment or even no treatment at all, is considered to be a factor with a poor prognosis.

Regarding the spinal tap, the cerebrospinal fluid appeared opalescent in the three patients who tested positive for *R. conorii*. Additionally, the cytology test revealed a mixed cellular profile in these three patients. Based on these findings, the opalescent appearance and the mixed cell profile may indicate the presence of a co-infection (in this case, with *R. conorii*) in patients presenting with meningeal syndrome.

In 2019, a study was conducted in South Africa to assess the laboratory diagnostic capacity for Spotted Fever Rickettsiosis. The study reported that there was no standardised testing platform or interpretation for serological tests across the various laboratories involved. Additionally, PCR tests were less frequently available. This lack of standardisation and availability poses a significant challenge for confirming diagnoses and managing cases, particularly given the multitude of potential differential diagnoses.¹²

In addition, further studies also took into account the Rickettsial disease. In Western Algeria, Oran, the first cases of MSF were clinically diagnosed for the first time in 1993, and since that time, the number of cases has steadily increased to reach 134 in 2004. Mouffok *et al.* in 2006, conducted a study in Western Algeria in order to present the descriptive clinic and epidemiology to identify more severe forms, the presence of multiple eschars, and different rickettsial strains caused the disease, where they found 63 positive IFA Rickettsial serology from out 104 sera, and they also reported that all the patients included in the study had fever, and 44% of them presented underlying diseases, and 72% of the population study have a Maculopapular rash.⁸ All the same, Mouffok *et al.*, in 2009, reported 24 positive IFA serology for *R. conorii* (24/36), belonging to Algerian children's patients. These results are in concordance with ours in terms of the clinical expression of Rickettsial disease and especially the presence of an underlying infection.¹³ In addition, Mouffok *et al.*, in 2011, evaluated the advantage of skin swab samples (Black Spot) for the diagnosis of Rickettsial diseases in Algeria by a molecular detection (qPCR) of the *RC0338* gene which is specific to *Rickettsia* spp; they obtained 63.4% of positive samples, these results confirmed that the efficiency of the skin swab sampling to diagnose the Rickettsioses, and also confirmed the presence of the Rickettsial pathogen in Algeria.¹⁴ Another study was conducted by Mokrani *et al* in 2012, aiming to have a prevalence of Rickettsioses in Febrile Exanthemas in Eastern Algeria, where they calculated 12.96% of positive IFA serology confirmed by Western Blot within 5 cases of *R. conorii*, 2 cases of *R. felis* and 4 cases of *R. typhi*, the left 03 cases belonged to other *Rickettsia* species (*R. aeschlimannii*).⁶ In a common epidemiological study of *Rickettsia felis* and Malaria in Africa, Mediannikov *et al.* in Africa (2013) worked for a cohort of African patients: 266 patients from Western Algeria, 183 patients from Tunisia and 48 patients from Morocco, and also 400 patients from Marseille as control patients. They reported 02 positive blood qPCR for *R. conorii* and 01 positive blood qPCR for *R. conorii* in Algerian and Moroccan patients, respectively. However, even Tunisia and Marseille have no positive case of *R. conorii*. These results confirm the linear relationship between Rickettsioses and other underlying infections (Malaria in this case), as we reported in our study between Rickettsioses and Meningeal Syndrome.

Nevertheless,² Znazen *et al.*, in 2006, in Tunisia, reported 86 positive IFA serology for *R. felis* confirmed by Western Blot from out 638 patients' sera tested.¹⁵ In addition, in Greece, Chochlakis *et al.*, in 2017, conducted a study aimed at improving testing of tick-borne diseases; they got 28 positive IFA for *R. typhi* and eight positive IFA for *R. felis*. Rickettsial diseases remain a

Table 3. Summarize of the study’s results conducted in Algeria to identify the borne-vector of *Rickettsia* species.

Authors	Vectors	qPCR gene targeted	Results	Rickettsia species
(16)	Ticks: - <i>Hyalomma marginatum</i> <i>Rhipicephalus sanguineus</i>	<i>gltA</i> , <i>OmpA</i> genes	21 positive qPCR of out 78	<i>R. conorii</i> <i>R. aschlimanii</i> <i>R. massilia</i> <i>R. helvetica</i>
(17)	Ticks: - <i>Ixodes ricinus</i> - <i>Dermacentor marginatus</i>	<i>gltA</i> , <i>OmpA</i> genes	15 positive qPCR of out 32	<i>R. monacensis</i> <i>R. slovacica</i>
(18)	Soft Ticks: Acari: Argasidae	<i>gltA</i> gene	41 positive qPCR of out 154	Novel <i>Rickettsia</i>
(19)	Ticks: <i>Rhipicephalus sanguineus</i>	<i>gltA</i> gene	01 positive qPCR of out 115	<i>R. conorii</i>
(20)	Ticks: - <i>Rhipicephalus sanguineus sensu lato</i> - <i>Rhipicephalus bursa</i> - <i>Hyalomma scupense</i> - <i>Hyalomma excavatum</i> .	<i>gltA</i> , <i>OmpA</i> genes	29 positive qPCR of out 191	<i>R. aschlimanii</i> <i>R. slovacica</i>

born-vector disease, mainly transmitted by arthropods, such as ticks, fleas, body lice and mites.¹

Rickettsia spp are widely distributed across the Mediterranean region, a geographic area characterised by diverse ecological landscapes that facilitate the survival and dissemination of these obligate intracellular bacteria. The seroprevalence of *Rickettsia* spp. in Mediterranean countries varies widely, ranging from approximately 8% to 30%.¹⁶ In Greece, serological data indicate rates between 8% and 20%, and this is especially true in rural communities and among individuals with suspected rickettsial illnesses.¹⁷ Moreover, in Turkey, studies have reported seroprevalence rates around 15–25%, particularly among livestock handlers and rural residents, which highlights occupational exposure risks.¹⁸ Similarly, in southern Italy and Spain, human seroprevalence can range from about 10% to 30%, with higher rates observed in populations with greater exposure to tick-infested environments or rural settings.¹⁹ Furthermore, in Morocco, the available data suggest seroprevalence rates around 5–10% among rural populations, reflecting a lower yet still notable level of exposure.¹⁶

Several studies have been conducted in Algeria to determine the various vectors and reservoirs of the Rickettsioses disease. **Table 3** summarises the results of these studies to identify the borne vector of *Rickettsia* species in Algeria.

The previous description of **Table 3** suggests the circulation of many *Rickettsia* spp in Algeria, transmitted mainly by ticks, which remain very important vectors of Rickettsial diseases, especially if they are transported by animal species where humans live.

Globalisation and climate change are factors that can contribute to the increased transmission of rickettsioses by ticks. Rising temperatures can enhance tick aggressiveness and their propensity to bite humans, while on the other hand, they can lead to infections

occurring at atypical times of the year. Furthermore, the growing trend of travelling to increasingly exotic destinations raises the likelihood of exposure to ticks that transmit diseases, including rickettsioses.²⁵

Conclusion. Based on serological findings and the reported clinical and epidemiological antecedents, there is clear evidence of the existence of human Rickettsial diseases in Algiers, especially with underlying infection. As we explained previously, both rickettsial diseases and meningeal syndromes could simultaneously infect humans, and the clinical expression reflects the severity of the pathogens in question. These findings would provide more attention for the infectious disease specialists in front of a confirmed Meningeal Syndrome, which may have a clinical similarity with the Rickettsial diseases, especially in countries where the hematophagous arthropod-borne zoonosis is highest. Moreover, Polymorphic diseases should, therefore, be suspected even in the absence of a rash.

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Ethics approval. The medical committee has approved the collection of samples from the patients included in this study. In addition, all patients provided consent to be included in the study, allowing us to collect interview information and blood samples. Clinical data were obtained through a standardised questionnaire, which included clinical information, contact with animals, and health history.

Author contributions. GH conceptually designed the strategy for this study, GH and AN collected samples, GH analysed the samples in the laboratory, IT and NI

advised on statistical analyses, GH, AN, and TS drafted the manuscript, and BI and NB analysed the data and corrected the manuscript.

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