Epidemiologia de les rickettsiosis del grup de les febres tacades a Catalunya. Estudi específic de la *Rickettsia massiliae* Bar 29. Eficàcia de les tècniques diagnostiques

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Ticks and Tick-borne Diseases 9 (2018) 629–631

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Detection of *Rickettsia massiliae*/Bar29 and *Rickettsia conorii* in red foxes (*Vulpes vulpes*) and their *Rhipicephalus sanguineus* complex ticks

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ARTICLE INFO

Keywords:
R. massiliae/Bar29
R. conorii
Red foxes (*Vulpes vulpes*)
Rh. sanguineus complex ticks

ABSTRACT

To determine the prevalence of exposure to *Rickettsia massiliae*/Bar29 and *Rickettsia conorii* in wild red foxes, we collected blood samples and ticks from 135 foxes shot in different game reserve areas in Catalonia. To detect SFG rickettsia in *Rhipicephalus sanguineus* complex ticks collected from the foxes, we used real-time polymerase chain reaction (PCR) to screen for *ompA* gene and a tick-borne bacteria flow chip technique based on multiplex PCR. Serum samples were positive for antibodies against spotted fever group (SFG) rickettsiae in 68 (50.3%). Molecular techniques identified *R. massiliae* in 107 ticks, *R. aeschlimannii* in 3 ticks, and *R. slovaca* in one tick; no *R. conorii* was identified in any of the ticks analyzed. We conclude that red foxes can carry ticks with SFG rickettsia.

1. Introduction

Rickettsia massiliae/Bar 29 and *Rickettsia conorii* are spotted fever group (SFG) rickettsiae transmitted by *Rhipicephalus sanguineus* complex ticks; this taxon includes the tropical and temperate clades, *Rh. leporis* and *Rh. turanicus* (Zemtsova et al., 2016). *Rh. sanguineus* sensu lato is the main vector of *R. conorii* (Brouqui et al., 2007) *Rickettsia massiliae* was first isolated from *Rh. turanicus* (Beati and Raoult, 1993); *R. massiliae*/Bar29 was later isolated from *Rh. sanguineus* in Catalonia (Beati et al., 1996). For years, *R. conorii* was considered the only causative agent of Mediterranean spotted fever, but molecular techniques have identified other SFG rickettsiae involved in human rickettsiosis (Blanda et al., 2017). The role of *R. massiliae* as a human pathogen was not confirmed until 2006, when it was first isolated from a patient (Vitale et al., 2006). Worldwide, few cases of *R. massiliae* infection in humans have been confirmed (Cascio et al., 2013; Garcia-Garcia et al., 2010; Zaharia et al., 2016); all were diagnosed by polymerase chain reaction (PCR) and DNA sequencing, but the pathogen was isolated in only one case (Vitale et al., 2006).

The eco-epidemiology of SFG rickettsiae involves a domestic and a wild cycle, both interconnected by ticks. The dog is a sentinel of *R. conorii* infection (Ortuño et al., 2009) and also has a role as a reservoir of *R. conorii* infection (Levin et al., 2012). However, co-feeding transmission could be sufficient for the circulation of rickettsiae in the vector population (Levin et al., 2014). Red foxes are frequently exposed to different arthropod vectors coming from their prey or from other hosts living in the same environment, and they represent an excellent sentinel of vector-borne diseases (Aguirre, 2009) and a possible source of infection for domestic animals and humans (Torina et al., 2013). The objectives of this study were to determine the prevalence of antibodies to *R. massiliae*/Bar29 and *R. conorii* in a survey of red foxes, and to identify SFG rickettsiae in *Rh. sanguineus* complex ticks removed from them.

2. Material and methods

Blood samples and ticks were collected from foxes shot by licensed hunters in several game reserve areas in Catalonia within the framework of an official fox population control program. Necropsies were done on all foxes, and blood samples were collected by intracardiac puncture. We recorded foxes' age, sex, health status based on teeth structure, and presence of ticks.

2.1. Serology

IgG antibodies against *R. conorii* and *R. massiliae*/Bar29 were detected by micro-immunofluorescence antibody (micro-IFA) test using an anti-dog IgG-FITC antibody produced in rabbits. *R. conorii* antigen

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https://doi.org/10.1016/j.ttbdis.2018.02.002
Received 29 March 2017; Received in revised form 1 February 2018; Accepted 2 February 2018
Available online 08 February 2018
1877-959X/

was provided by Dr. Rita de Sousa, *Instituto Nacional de Saúde Doutor Ricardo Jorge*, I.P. (INSA) *Departamento de Doenças Infecciosas* (DDI), Portugal. *R. massiliae*/Bar29 antigen was obtained from a strain previously isolated from *Rh. sanguineus* in our region (Beati et al., 1996) cultured on Vero cells at 32 °C in an atmosphere containing 5% CO_2. Both antigens were applied to a 10-well microscope slide (BioMérieux, Marcy l'Etoile, France), and the slides were air-dried and fixed in methanol-acetone, as described by Wächter et al. (2015).

Briefly, 20 µL of fox serum was initially screened at 1:40 in PBS-5% BSA, incubated in a humidified chamber at 37 °C for 30 min, and washed twice in PBS and once in water. Binding serum samples were detected using a fluorescein isothiocyanate-labelled anti-dog IgG (Sigma-Aldrich, Madrid, Spain) at the dilution recommended by the manufacturer. The slides were incubated and washed as described above. Once dried, the slides were mounted with buffered glycerol and examined with a fluorescent microscope (400×). Positive serum samples were then titrated in a twofold dilution until the definitive titer was obtained. The highest dilution at which distinct and specific fluorescence was seen was scored as the end-point titer for the serum sample.

2.2. Ticks

When present, ticks were removed and identified using taxonomic keys (Estrada-Peña et al., 2004); ticks identified as *Rh. sanguineus* complex ticks were analyzed with molecular techniques.

2.2.1. DNA extraction

Ticks were cleared in 100 µL brain-heart infusion extraction. DNA was extracted using the MasterPure DNA purification kit (Epicentre, Madison, USA) according to the manufacturer's instructions.

2.2.2. PCR and DNA sequencing

Rh. sanguineus complex ticks were analyzed by two techniques: real-time PCR targeting the gene *ompA* and multiplex PCR.

To detect the *ompA* gene, we used *Rickettsia*-specific PCR assays previously designed by our laboratory to amplify a fragment of 316 nucleotides within the *ompA* gene (314-FOR: 5′-GGGCATTTACTTACG GTGGTGAT-3′; 630-REV: 5′-CTTTGACGGAGCTGCAGATTGTAT). We used a Sensimix dU kit (Bioline Reagents, London, UK) that included Uracil DNA Glycosylase (UNG) and deoxyuracil triphosphate (dUTP) to avoid carry-over contamination. 0.2 µM of each primer was used. PCR samples were prepared in a UV-sterilized workstation, and real-time PCR assays were performed with a 7500 thermocycler (Applied Biosystems, Foster City, CA, USA). All assays included two negative controls (PCR reagents and DNA-free water) and one positive control (DNA from *R. conorii* or *R. massiliae*/Bar29). Each sample was assayed twice. Amplification products were purified by ExoSAP-IT (GE Healthcare Life Sciences, Buckinghamshire, UK) and directly sequenced using forward and reverse primers on a 3130 Genetic Analyzer with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequences obtained were compared with those in the GenBank nucleotide database using the Nucleotide BLAST program.

A tick-borne bacteria flow chip kit (Master Diagnóstica, Granada, Spain) was also used to detect bacterial DNA in ticks by multiplex PCR followed by reverse dot blot hybridization over a membrane with specific probes for different bacteria transmitted by arthropods. The kit detects pathogenic bacteria belonging to the genera *Anaplasma, Ehrlichia, Borrelia, Bartonella, Coxiella, Rickettsia* and *Francisella*. In this case, amplified products were sequenced by the manufacturer of the kit.

Statistical analysis was performed using the chi-square test on Statcalc, Epi-Info v.6 program computer package (CDC, Atlanta, USA). Differences were considered statistically significant when $P < 0.05$.

3. Results

We analyzed blood samples from 135 foxes (69 male and 66 female); 27 were considered as young (≤1 year) and 108 were considered as adults (> 1 year); most (n = 126) were in good health, but nine were emaciated and eight of these had mange.

Table 1
Distribution of serological titers to *R. conorii* and to *R. massiliae*/Bar29 detected in foxes.

R. massiliae/Bar29	*R. conorii*					
	Neg	1:40	1:80	1:160	1:320	1:640
Neg	67	3	3	–	–	–
1:40	23	3	–	–	–	–
1:80	8	3	4	2		–
1:160	2	–	7	3	1	–
1:320	1	1	1	–	2	–
1:640	–	–	1	–	–	–

3.1. Serology

Serum samples were considered positive for SFG rickettsiae when either of the two antigens showed fluorescent patterns at 1/40 dilution. A total of 68 (50.3%) samples were positive for SFG rickettsiae. These included 62 (45.9%) positive for *R. massiliae*/Bar29 (titers 1/40–1/640) and 34 (25.2%) positive for *R. conorii* (titers 1/40–1/320); 34 foxes were positive for *R. massiliae*/Bar29 and negative for *R. conorii*, but only 6 were positive for *R. conorii* and negative for *R. massiliae*/Bar29 (Table 1).

There were no differences in sex, age, or presence of ticks between foxes with positive serum samples and those with negative serum samples.

3.2. Ticks

Adult *Rh. sanguineus* complex ticks were identified on 84 foxes, adult Ixodes ricinus ticks on two foxes, and adult Dermacentor marginatus ticks on one fox. Moreover, two nymphs of Ixodes spp. ticks were also detected on one fox.

We analyzed 137 *Rh. sanguineus* complex ticks from 32 foxes. We detected *Rickettsia* DNA in 80 ticks by real-time PCR and in 114 ticks by multiplex PCR. The species was identified in 111 cases: *R. massiliae* (identity range > 98%) (n = 107), *R. aeschlimannii* (identity range 99%) (n = 3), and *R. slovaca* (n = 1); in the remaining 3 cases, only the genus *Rickettsia* was identified. All samples that were positive by real-time PCR were also positive by multiplex PCR. *R. conorii* was not identified in any ticks. Among ticks infected with *R. massiliae, Bartonella henselae* was also detected in 19, other *Bartonella* spp. in 2, and *Borrelia* spp. in 12. In 3, *R. massiliae*, *Bartonella* spp., and *Borrelia* spp. were detected.

We observed no statistical differences when comparing fox seropositivity and *Rickettsia*-infected ticks. *R. massiliae*-infected ticks were removed from 19 seropositive foxes and 10 seronegative foxes; no *Rickettsia* was detected in ticks removed from 2 seronegative foxes and one seropositive fox.

4. Discussion

We detected antibodies to *R. conorii* and *R. massiliae*/Bar29 in red foxes from Catalonia. To the best of our knowledge, this is the first survey to detect antibodies against these SFG rickettsiae in red foxes. In nature, red foxes are exposed to several tick species that may be infected with more than one *Rickettsia* spp. Considering that strong cross-reactivity is well documented among SFG *Rickettsia* spp., we can only conclude that foxes were exposed to SFG rickettsiae. However, cutoff values in the micro-IFA procedure are not completely standardized in wild animals or domestic animals. In dogs, some authors used a cutoff screening dilution of 1/128 (Barrett et al., 2014; Wächter et al., 2015), while others used 1/16 (Levin et al., 2014).

The role of red foxes in the eco-epidemiology of SFG rickettsiae is

unknown, since no *Rickettsia* spp. have been detected by molecular techniques in foxes (Boretti et al., 2009; Torina et al., 2013). Unfortunately, we were unable to analyze fox blood samples by molecular biology, and this is a limitation of this study.

Other authors have identified *R. massiliae* in *Rh. sanguineus* ticks collected from foxes (Marié et al., 2012; Chisu et al., 2016). In our study, *R. massiliae* was the most common SFG rickettsia detected in *Rh. sanguineus* complex ticks analyzed, and *R. conorii* was not detected in any. These findings agree with those reported elsewhere (Marié et al., 2012; Socolovschi et al., 2012; Rose et al., 2017). Milhano et al. (2014) observed the transstadial and transovarial survival of *R. massiliae* in *Rh. sanguineus* without cytopathic effects in the vector cells. In contrast, *.R. conorii* subsp. *israelensis* have deleterious effects in *Rh. sanguineus* sensu lato ticks (Levin et al., 2009), and this might explain their low prevalence in nature (usually less than 1%) (Rose et al., 2017).

In this study, *R. aeschlimannii* was identified in three *Rh. sanguineus* complex ticks. This rickettsia was first isolated in *Hyalomma marginatum* collected in cattle from Morocco (Beati et al., 1997). In fact, *Hyalomma* ticks are considered its main vector and reservoir. Recently, *R. aeschlimannii* was detected in *R. turanicus* collected from sheep in China (Wei et al., 2015). Phylogenetically, this rickettsia belongs to the SFG and is grouped with *R. massiliae*, *R. massiliae*/Bar29, *Rickettsia rhipicephali*, and *Rickettsia montanensis* (Parola et al., 2005).

To detect tick-borne bacteria other than rickettsia, we used multiplex PCR. Quarsten et al. (2015) compared multiplex PCR based on a tick-borne bacteria flow-chip system and corresponding real-time PCR protocols, concluding that real-time PCR was slightly more sensitive for detecting *Borrelia* spp and *Anaplasma phagocytophilum*. In the present study, we used both molecular methods to screen all ticks. Multiplex PCR was more sensitive than real-time PCR for *Rickettsia* spp., and all positive multiplex PCR results were confirmed by sequencing. Moreover, multiplex PCR detected DNA of other pathogens, such as *Borrelia* and *Bartonella* in *Rh. sanguineus* complex ticks. Further studies are needed to clarify the vector role of *Rh. sanguineus* complex ticks in the transmission of rickettsia, as well as to identify which species of *Bartonella* and *Borrelia* are detected in *Rh. sanguineus* complex ticks.

Red foxes are among the most common wild carnivores in Europe, and they are very well adapted to suburban and rural environments. In the eco-epidemiology of SFG rickettsiae, red foxes may act as carriers of ticks infected with rickettsiae, as occurs in other natural hosts, such as dogs in the domestic cycle of *R. conorii* infection (Ortuño et al., 2009) or ruminants and wild-boar in that of *R. slovaca* (Ortuño et al., 2007; Ortuño et al., 2012).

5. Conclusions

Red foxes are exposed to SFG rickettsiae. *R. massiliae*/Bar 29 but not *R. conorii* was detected in *Rh. sanguineus* complex ticks removed from red foxes. Our results suggest that red foxes may be carriers of ticks infected with SFG rickettsiae.

Conflict of interest

All of the authors declare no conflict of interests related to this article.

Acknowledgment

This study was supported by Ministerio de Sanidad y Consumo, Spain (grant FIS PI11/02249).

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Eur J Clin Microbiol Infect Dis (2006) 25:541–543
DOI 10.1007/s10096-006-0176-1

BRIEF REPORT

Serological evidence of human infection with rickettsial strain Bar29 in Catalonia, northeastern Spain

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Published online: 18 July 2006

Mediterranean spotted fever (MSF) is an endemic disease in Catalonia, northeastern Spain. The causative agent is *Rickettsia conorii*, which is transmitted by the dog tick *Rhipicephalus sanguineus* in the Mediterranean area [1, 2]. However, it is strongly suspected that another spotted fever group (SFG) rickettsial strain could be implicated in some cases of MSF diagnosed in Catalonia [3, 4].

In 1990, Mtu1 and Mtu5 were isolated from *Rhipicephalus turanicus* ticks collected in the south of France. Both were new serotypes related to the SFG rickettsiae [5]. Mtu1 was named *Rickettsia massiliae* and proposed as a new SFG *Rickettsia* species [6]. Strains related to *R. massiliae* have been identified throughout the world [7]. In Catalonia, a new SFG rickettsial strain (Bar29) was isolated by our group in 1996 from *R. sanguineus*; it is identical to the Mtu5 strain and closely related to *R. massiliae* [3]. *R. massiliae* Bar29 belongs to the *R. massiliae* group of SFG that includes *R. massiliae*, *Rickettsia aeschlimannii*, *Rickettsia montanensis* and *Rickettsia rhipicephali* [7].

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A previous study we conducted using sera from Catalonian patients suggested *R. massiliae* Bar29 could be implicated in cases of MSF diagnosed in our region [4]. Currently, *R. massiliae* and *R. aeschlimannii* are recognized as human pathogens [7, 8]. The aim of the study presented here was to determine the seroprevalence of *R. massiliae* Bar29 in a representative population of Catalonia and to identify possible risk factors for infection.

The study was performed in a predominantly urban area near the coast in northeastern Spain. Between September 1993 and January 1994, 211 subjects who had received attention at Sabadell's Hospital were chosen to participate in a study searching for antibodies to *R. conorii* and *Borrelia burgdorferi*. The sample size had been estimated taking into account overall prevalences of 11 and 3% of antibodies to *R. conorii* and *B. burgdorferi*, respectively (given an alpha error of 0.05). The sample included adults undergoing minor surgery and children who visited the Pediatric Emergency Service. Taking into account the actual population, the study population was stratified by age and residential area. After informed consent was obtained, a serum sample was collected. Age, sex, demographic area, occupation, contact with dogs, contact with wild animals, contact with farm animals, and history of MSF were surveyed.

One milliliter of blood was collected in tubes containing heparin. The sample of heparinized blood was sedimented and the supernatant (0.5 ml) was collected and stored at −80°C. Human sera were evaluated using an indirect immunofluorescence assay (IFA) for IgG antibodies [9]. Antigen was obtained from the *R. massiliae* Bar29 strain previously isolated from ticks and cultured in our laboratory [3]. Titers ≥1/40 were considered positive. Statistical analysis was performed using the chi-square test, Fisher's

exact test, or the Student *t* test. A *p* value of <0.05 was considered significant.

Eighteen (8.5%) serum samples were found with antibodies against *R. massiliae* Bar29. Positive sera titers ranged from 1/40 to >1/1,280 and nine (4.24%) samples had titers ≥1/80. Nine of the positive samples had an IgG titer of 1/40, seven a titer of 1/80, one a titer of 1/320, and one a titer of >1/1,280. The relationships between the seropositive rate and the surveyed items are shown in Table 1.

The association between *R. massiliae* Bar29 strain seropositivity and contact with dogs (pet or stray dogs) was statistically significant (p=0.018, OR 3.59 [1.29–10.05]). While the association between seropositivity and contact with pet dogs was not significant, contact with stray dogs was significantly related to *R. massiliae* Bar29 infection, probably because they tend to be more infested than pet dogs. Seroprevalence in dogs has been considered a marker of infection with *R. conorii*. Therefore, the presence of *R. massiliae* Bar29 in dogs and ticks, as well as the roles these carriers play in the *R. massiliae* Bar29 infection cycle, should be surveyed.

Table 1 Demographic information from individuals in Catalonia tested for Bar29 strain infection

Variable	No. (%) of subjects (n=211)	No. (%) positive (n=18)
History of MSF	0 (0)	0 (0)
Mean age in years±SD	33.92±23.19	49.89±18.84
Age in years		
0–14	51 (24.2)	1 (5.6)
15–29	49 (23.2)	1 (5.6)
30–44	42 (19.9)	4 (22.2)
45–64	44 (20.9)	8 (44.4)[a]
≥65	25 (11.8)	4 (22.2)
Sex		
Male	114 (54)	13 (72.2)
Female	97 (46)	5 (27.8)
Residential area[b]		
Urban	141 (66.8)	13 (72.2)
Semi-rural	57 (27)	4 (22.2)
Rural	13 (6.2)	1 (5.6)
Occupation (>18 years)	149	16
Student	11 (7.4)	0 (0)
Retired	25 (16.8)	4 (25)
Housewife	36 (24.2)	4 (25)
Worker	50 (33.6)	3 (18.8)
Unemployed	26 (17.4)	4 (25)
Unknown	7 (4.3)	1 (6.3)
Animal contact		
Pet dog	34 (16.1)	6 (33.3)
Stray dog	4 (1.9)	2 (11.1)[c]
Farm animal	7 (3.3)	0 (0)
Wild animal	2 (0.9)	0 (0)

[a] p=0.028, OR=3.49 (1.29, 9.46)
[b] Defined by number of inhabitants of municipalities: rural <5,000; semi-rural 5,000–50,000; urban >50,000
[c] p=0.034, OR=12.73 (1.67, 96.88)

A statistically significant association between seropositivity and age was observed. The mean age of seropositive subjects was 49.89±18.84 years (range 3–82 years), whereas the mean age of seronegative subjects was 32.43±23.04 years (range 0–91 years), p=0.001. Seropositivity was significantly more prevalent in 45–64-year-old subjects compared with patients younger than 45 or older than 64 years [p=0.028, OR 3.49 (1.29–9.46)]. In the seroprevalence survey we previously carried out in southern Spain [10], the same association was observed. It also held true for *R. conorii* in both the northeast [2] and the south [10] of Spain; those studies showed the mean age of subjects with antibodies against *R. conorii* was higher than the mean age of seronegative patients. This fact could be explained by the longer exposure time experienced by older people.

In our previously reported serological study of *R. conorii* carried out with the same sera [2], ten (4.7%) serum samples had antibodies against *R. massiliae* Bar29 exclusively; four serum samples reacted against *R. massiliae* Bar29 and *R. conorii* at the same titer levels (1/40, 1/40, 1/80, 1/80); two sera showed higher titers against *R. massiliae* Bar29 (1/320 vs 1/80 and >1/1,280 vs 1/640); and in two other samples titers against *R. conorii* were higher (1/160 vs 1/40 and 1/320 vs 1/80). These last two sera should not be considered positive for *R. massiliae* Bar29. These results indicate *R. massiliae* Bar29 may be present in our area and its seroprevalence would range from 4.7 to 7.6%.

This is the first report of human reactivity to *R. massiliae* Bar29 in northeastern Spain, and it indicates *R. massiliae* Bar29 could be implicated in human infection in Catalonia, in addition to *R. conorii*. *R. massiliae* Bar29 seems to be more prevalent in 45–64-year-old people. Contact with dogs seems to be a risk factor for infection with *R. massiliae* Bar29.

A serologic survey is a relatively easy way to get preliminary data on whether a disease occurs in a region, and our results support the suspicion that the *R. massiliae* Bar29 strain could be implicated in an indeterminate percentage of MSF cases diagnosed in Catalonia [4]. Additional studies with isolation and characterization of this strain in patients from our region would confirm *R. massiliae* Bar29 human infection in Catalonia.

Acknowledgments This study was supported by Ministerio de Sanidad y Consumo grant FIS01/1082. "Red Española de Investigación en Patología Infecciosa—REIPI" (Spain) has partially supported this work. The study complies with the current laws of Spain.

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Ten Years' Experience of Isolation of *Rickettsia* spp. from Blood Samples Using the Shell-Vial Cell Culture Assay

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ABSTRACT: Two strategies to improve the efficacy of the shell-vial method for *Rickettsia* were analyzed. Blood samples from 59 patients with Mediterranean spotted fever (MSF) were examined using the shell-vial technique. (i) DNA from positive lenses was obtained when they were contaminated. (ii) Blood sample from one patient was cultured in 17 shell-vials. *R. conorii* was identified in four cases by polymerase chain reaction (PCR)-RFLP. Three of these were obtained from cells adherent to lenses and the fourth one by using total patient blood sample. *Rickettsia* isolation using all blood samples as well as DNA from shell-vial lenses could be useful in the study of rickettsial infections.

KEYWORDS: shell-vial assay; *Rickettsia*; MSF (Mediterranean spotted fever)

INTRODUCTION

The main problem in diagnosing rickettsial disease is because its characteristics of intracellular microorganism makes it difficult to use conventional culture methods. Thus the etiological diagnosis, epidemiological studies, and effectiveness of the treatment are difficult to assess. The specific diagnosis by culture needs a cellular culture medium and the traditional methods are slow and cumbersome and can only be done in special research laboratories. For many years different researchers have adapted the shell-vial culture assay (e.g., viruses) used for the diagnosis of other organisms virus, to *Rickettsia* with various results.[1–6] The shell-vial method combines the centrifugation of

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Ann. N.Y. Acad. Sci. 1078: 578–581 (2006).
doi: 10.1196/annals.1374.115

the sample on the cellular line and the development by immunofluorescence (IFI). In this way we can decrease the time needed for the culture making this method more adaptable to conventional laboratories and allowing in theory a quick diagnosis between 48–72 h. Apart from its possible use for the diagnosis of the disease, this method allows the identification of different *Rickettsia* species for epidemiological studies.[7]

In our laboratory we have been using this method together with specific serological techniques in the study of *Rickettsia* in patient's samples since 1994.

AIM

We presented the results obtained using the shell-vial technique for the detection and isolation of rickettsial strains from blood samples of patients with presumptive diagnosis of Mediterranean spotted fever (MSF). Furthermore, we evaluated two strategies to improve the efficacy of the shell-vial culture method in our laboratory.

PATIENTS AND METHODS

During the period 1994–2003, blood samples from 59 adult patients clinically diagnosed with MSF and with seroconversion to *R. conorii* were cultured by the shell-vial technique using MRC5 cells incubated at 32°C in Eagle's minimal essential medium containing 10% fetal bovine serum and 2 mM L-glutamine (Innogenetics, Barcelona, Spain). From each patient three shell-vial assays were carried out. The time of incubation varied between 4 and 15 days. The supernatants of the shell-vial positive by IFI indirect were inoculated in the same line cells under the same conditions. We used two strategies to improve the results: (i) DNA was obtained from cells adherent to shell-vial lenses positive by IFI whose cultures were contaminated. DNA was extracted by using a QIAamp DNA Mini Kit (Qiagen GmbH, Germany), as recommended by the manufacturer. (ii) In another case, total blood sample (8.5 mL) from one patient in 2004 was cultured in 17 shell-vials in order to increase the sensitivity. Then all the supernatants were cultured. To control the infection Gimenez staining was used.

To identify the SFG rickettsia species seen by IFI and by Gimenez staining, polymerase chain reaction (PCR)/RFLP was used.[8] PCR amplification was performed by using oligonucleotide primer pairs Rp CS877p and Rp CS.1258n generated from the citrate synthase gene of *R. prowazekii*, Rr 190.70p and Rr 190.602n generated from the 190-kDa antigen gene of *R. rickettsii*, and BG1-21 and BG2-20 generated from the 120-kDa antigen gene of *R. rickettsii*.[9,10] PCRs were carried out using a Perkin-Elmer 9600 thermocycler (Roche Diagnostics, Norwalk, CT), according to the protocol described by Regnery *et al*.[10]

The amplified products were analyzed on a 1.8% agarose gel (Bio-Rad Laboratories, Hercules CA) in 0.5 × Tris-borate-EDTA (TBE) buffer (AppliChem GmbH, Darmstat, Germany). As positive control, purified DNA from *R. conorii* (a kind gift from D. Raoult, Unité des Rickettsies CNRS UMR 6020, IFR 48, Faculté de Médecine de Marseille, Université de la Méditerranée, Marseille, France) was used. As a negative control destilled water was included in the same way as the DNA samples.

Amplified products were digested with *Alu*I, *Rsa*I, and *Pst*I restriction endonucleases, according to the protocol described by Eremeeva *et al*.[9] Electrophoretic separation was performed in a gel consisting of 1.8% agarose in TBE buffer.

The DNA fragments were visualized by ethidium bromide staining, and fragment sizes were compared with the sizes from a DNA Molecular Weight Marker VI (Boehringer Mannheim, GmbH, Germany).

RESULTS

From 59 patients (1994–2003) suspected of having MSF, only 5 had positive shell-vial cultures easily recognized by direct IFI staining. Detection of *Rickettsia* spp. was achieved on the first stained shell-vial (day 4) in five cases. One out of five positive shell-vials were not possible to be cultured. Another one was identified by partial *omp*A sequence as *R. conorii* (Malish 7 strain).[1] The remaining three positive shell-vials were contaminated.

Strategy A: Although we could not obtain the strains in three cases because they were contaminated, we could amplify *gtl*A, *omp*A, and *omp*B DNA fragments from DNA extracted from the cells adherent to shell-vial lenses.

Strategy B: A total of 4 of the 17 shell-vials from one patient in 2004 were strongly positive, 6 were weakly positive, and the rest negative. However, when the supernatants were cultured, we isolated *Rickettsia*-like organisms in all cases. We also amplified specific rickettsial DNA fragments. In both strategies, all amplified fragments had the same enzyme restriction profiles as the control *R. conorii*.

CONCLUSIONS

The shell-vial culture is the most suitable method to obtain and identify *Rickettsia* strains from patient blood samples, because it is an easy and fast methodol. To improve its lack of sensibility we can perform PCR of contaminated shell-vials lenses or using up all the extracted blood samples in shell-vial cultures, which in our cases have been shown to be useful. Any way to improve the effectiveness of *Rickettsia* culture will be useful to increase our knowledge of the epidemiologic situation of *Rickettsia* in any country.

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Ticks and Tick-borne Diseases 7 (2016) 457–461

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Short communication

Shell-vial culture, coupled with real-time PCR, applied to *Rickettsia conorii* and *Rickettsia massiliae*-Bar29 detection, improving the diagnosis of the Mediterranean spotted fever

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ARTICLE INFO

Article history:
Received 22 April 2015
Received in revised form 5 January 2016
Accepted 18 January 2016
Available online 19 January 2016

Keywords:
Mediterranean spotted fever
Rickettsia conorii
Rickettsia massiliae-Bar29
Shell-vial assay
Real-time PCR

ABSTRACT

Rickettsia conorii and *Rickettsia massiliae*-Bar29 are related to Mediterranean spotted fever (MSF). They are intracellular microorganisms. The Shell-vial culture assay (SV) improved *Rickettsia* culture but it still has some limitations: blood usually contains low amount of microorganisms and the samples that contain the highest amount of them are non-sterile. The objectives of this study were to optimize SV culture conditions and monitoring methods and to establish antibiotic concentrations useful for non-sterile samples.

12 SVs were inoculated with each microorganism, incubated at different temperatures and monitored by classical methods and real-time PCR. *R. conorii* was detected by all methods at all temperatures since 7th day of incubation. *R. massiliae*-Bar29 was firstly observed at 28 °C. Real-time PCR allowed to detected it 2–7 days earlier (depend on temperature) than classical methods. Antibiotics concentration needed for the isolation of these *Rickettsia* species from non-sterile samples was determined inoculating SV with *R. conorii*, *R. massiliae*-Bar29, biopsy or tick, incubating them with different dilutions of antibiotics and monitoring them weekly.

To sum up, if a MSF diagnosis is suspected, SV should be incubated at both 28 °C and 32 °C for 1–3 weeks and monitored by a sensitive real-time PCR. If the sample is non-sterile the panel of antibiotics tested can be added.

1. Introduction

Rickettsia conorii is the etiological agent of Mediterranean spotted fever (MSF). Although MSF is endemic in Mediterranean area, it has also been reported in Northern and Central Europe, Northern Africa, Middle East, and India (Parola et al., 2013). MSF is usually mild; however, it can be a severe and fatal disease. Its main vector is *Rhipicephalus sanguineus* (Parola et al., 2013). Since some MSF clinical cases showed differences related to severity or antibiotic sensitivity (Eremeeva et al., 2006), it was strongly suspected that another microorganism could be involved in some MSF cases (Cardeñosa et al., 2003). In 1990, *Rickettsia massiliae* was isolated from *Rhipicephalus turanicus* (Beati and Raoult, 1993). Afterwards, the strain Bar29 was isolated from *Rh. sanguineus* in our region (Beati et al., 1996). Although serological studies pointed to it could cause human infection (Bernabeu-Wittel et al., 2006; Cardeñosa et al., 2003; Cardeñosa et al., 2006), its role as a human pathogen was not confirmed until its isolation from a patient in 2006 (Vitale et al., 2006). *R. massiliae* is worldwide distributed (Beeler et al., 2011; Parola et al., 2013; Segura et al., 2014). Up to now, three human cases of *R. massiliae* infection have been described (Parola et al., 2013; Vitale et al., 2006), and a few strains have been isolated (Babalis et al., 1994; Beati and Raoult, 1993; Beati et al., 1996; Eremeeva et al., 2006; Milhano et al., 2010; Vitale et al., 2006).

Both *R. conorii* and *R. massiliae* belong to Spotted fever group rickettsiae (SFGR). They are Gram-negative, obligate intracellular microorganisms. When a MSF case is suspected, *R. conorii* infection is usually diagnosed by indirect immunofluorescence assay (IFA). This assay has three main limitations: it requires seroconversion and, thus, it can not be applied during the early stages of infection, cross-reactions among SFGR make difficult a correct identification

Abbreviations: FCS, fetal calf serum; FOV, field of view; IFA, Indirect immunofluorescence assay; MEM, Minimal essential medium; MSF, Mediterranean Spotted Fever; SFGR, Spotted fever group rickettsiae; SV, Shell-vial culture assay.

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http://dx.doi.org/10.1016/j.ttbdis.2016.01.008
1877-959X/

of the etiological agent (Babalis et al., 1994; Bernabeu-Wittel et al., 2006; Cardeñosa et al., 2006; Segura et al., 2014), and there is no commercial assay for testing antibodies against *R. massiliae.*

Culture can be applied during early stages, increases the number of microorganisms to be detected and allows to isolate and identify new strains. The Shell-vial culture assay (SV) was adapted to *Rickettsia* and it has been applying up to now (Vestris et al., 2003). Growth in SV is monitored by IFA or Gimenez staining (Babalis et al., 1994; Beati et al., 1996; Cardeñosa et al., 2000; Espejo-Arenas and Raoult, 1989; Marrero and Raoult, 1989; Milhano et al., 2010; Péter et al., 1990). This study was based on the hypothesis that application of real-time PCR as monitoring method may increase the SV sensitivity. In addition, since non-sterile samples are usually the most useful because they contain higher number of microorganisms, it is required to analyze which antibiotics can be used. Therefore, this study had two main objectives: to optimize SV culture conditions and monitoring methods for *R. conorii* and *R. massiliae*-Bar29 detection, and to analyze which antibiotics can be useful for their isolation from non-sterile samples.

2. Materials and methods

2.1. *Culture of* Rickettsia conorii *and* Rickettsia massiliae-*Bar29*

R. conorii and *R. massiliae*-Bar29, previously obtained by our group (Beati et al., 1996; Cardeñosa et al., 2000), were cultured in flasks seeded with Vero cells (African green monkey kidney epithelial cells; Inverness medical). Minimal essential medium (MEM) (Lonza, Basel, Switzerland), supplemented with 10% of fetal calf serum (FCS) (Lonza, Basel, Switzerland) and 2 mM of glutamine (Lonza, Basel, Switzerland), was used throughout this study. Both microorganisms were incubated at 32 °C. Growth was monitored by Gimenez staining. When infection was clearly observed, cell monolayers were scraped with glass beads. Gimenez staining of *R. conorii* showed microorganisms in the cytoplasm of 80–90% Vero cells and in the medium (25–50 microorganisms/field of view [FOV]). *R. massiliae*-Bar29 was observed in the cytoplasm of 70% of Vero cells and in the medium (10–15 microorganisms/FOV).

2.2. *Optimization of growth temperatures and evaluation of detection methods*

Culturing: In order to emulate the low concentrations of microorganisms usually found in blood samples, above mentioned cultures were diluted in MEM in order to have a minimum number of microorganisms in the inoculum. *R. conorii* was diluted 1:1000, and *R. massiliae*-Bar29 was diluted 1:300. No microorganism was observed in these dilutions by Gimenez staining. Each dilution was inoculated in 12 shell-vials (SV) seeded with Vero cells (100 μL/SV). For each microorganism, SV were incubated in quadruplicate at 28 °C, 32 °C, or 37 °C. After three days, the medium was changed to eliminate any microorganisms present in the inoculum.

Monitoring: Cultures were monitored every 3–4 days from the 7th day to the 29th day after inoculation. At each time, medium was replaced and cultures were monitored by Gimenez staining and molecular detection. Indirect immunofluorescence assay (IFA) was performed on the 9th, 15th, 22nd and 29th days.

In this experiment, monitoring by Gimenez staining consisted of an exhaustive search of microorganisms in up to 90% of fields of view, which was independently carried out by two researchers. The minimum and maximum numbers of bacteria for field of view were obtained. Vero cells observed by Gimenez staining were graded considering how full of *Rickettsia* they were. They were graded from value 0 (no *Rickettsia* were observed inside) to value 10 (Vero cells were completely full of *Rickettsia*).

For molecular detection, DNA was obtained from 0.5 mL of medium using Masterpure DNA purification kit (Epicentre, Madison, Wisconsin). DNA extractions were stored at −20 °C until use. DNA samples were tested in duplicate by *Rickettsia* spp. specific real-time PCR targeting the gene for 17 kDa protein (Jiang et al., 2004). Each PCR plate contained one positive control (DNA from *R. conorii* or *R. massiliae*-Bar29) and two negative controls (DNA-free water).

Indirect immunofluorescence assay (IFA) was performed as follows: SV coverslips were fixed with acetone. A human serum, which contained antibodies against *R. conorii* (1/1024), and *R. massiliae*-Bar29 (1/256), was diluted in PBS. It was diluted 1/128 considering the lowest titre. Coverslips were incubated with this dilution. Binding antibodies were detected using a fluorescein isothiocyanate-labeled anti-human IgG (Sigma–Aldrich Química, S.A., Madrid). The coverslips were examined with a fluorescence microscope at 400× and independently evaluated by two of the authors.

2.3. *Evaluation of antibiotics*

Antibiotics: Gentamicin, amphotericin B, and vancomycin were chosen because all together act against a wide range of Gram-negative and Gram-positive bacteria and Fungi. Medium was prepared with those concentrations described by Melles et al. (1999). In fact, medium (MEM + 10% FCS + 2 mM glutamine) containing 10 μg/mL gentamicin, 2.5 μg/mL amphotericin B, and 10 μg/mL vancomycin were prepared. This initial medium was diluted (using MEM + 10% FCS + 2 mM glutamine) as follows: 3:4 (7.5 μg/mL gentamicin, 1.88 μg/mL amphotericin B, and 7.5 μg/mL vancomycin), 1:2 (5 μg/mL gentamicin, 1.25 μg/mL amphotericin B, and 5 μg/mL vancomycin), and 1:4 (2.5 μg/mL gentamicin, 0.62 μg/mL amphotericin B, and 2.5 μg/mL vancomycin).

Culturing: Table 1 shows the design of the experiment. Shell-vials were inoculated with 100 μL/SV of *R. conorii* culture previous diluted (above mentioned), *R. massiliae*-Bar29 culture previous diluted (above mentioned), and a tick and a biopsy triturated in BHI. The tick was a *Dermacentor marginatus* that had been collected on a patient with an initial suspicious of Rickettsiosis, finally discarded. Biopsy was kindly obtained from Microbiology laboratory of our hospital and belonged to a patient without a Rickettsiosis diagnosis. Medium with antibiotics was added as it is shown in Table 1. All SV were incubated at 32 °C. On the 3rd day, mediums were changed by the same mediums without antibiotics.

Monitoring: Cultures were monitored weekly by Gimenez staining and IFA. Both methods were carried out using a drop of supernatant. They were evaluated independently by two of the authors.

3. Results

3.1. *Optimization of growth temperatures and evaluation of detection methods*

Data obtained is shown in Table 2. *R. conorii* grew at 28 °C, 32 °C and 37 °C. In fact, it was detected, by Gimenez and real-time PCR, in all SV incubated at each temperature after 7 days of incubation. All IFA assays were positive. At 32 °C, 25–45 microorganisms free in the supernatant for field of view as well as Vero cells 80–90% full of microorganisms were observed since the 12th day. However, similar amount of microorganisms were not observed until the 17th day at 28 °C and 37 °C.

R. massiliae-Bar29 grew at 28 °C and 32 °C, but it did not grow at 37 °C. At either 28 °C or 32 °C, one SV was positive whereas the other three ones were negative. *R. massiliae*-Bar29 was detected

Table 1
Design of the experiment on evaluation of antibiotics. Distribution of antibiotics concentrations and inoculum throughout shell-vials.

Inoculum	Number of SVs	Number SV/Antibiotics concentration[a]			
		T	3:4	1:2	1:4
Rickettsia conorii	12 SV	3 SV	3 SV	3 SV	3 SV
Rickettsia massiliae-Bar29	12 SV	3 SV	3 SV	3 SV	3 SV
Biopsy	4 SV	1 SV	1 SV	1 SV	1 SV
Tick	4 SV	1 SV	1 SV	1 SV	1 SV

[a] T (total): 10 μg/mL gentamicin, 2.5 μg/mL amphotericin B, 10 μg/mL vancomycin; 3:4: 7.5 μg/mL gentamicin, 1.88 μg/mL amphotericin B, 7.5 μg/mL vancomycin; 1:2: 5 μg/mL gentamicin, 1.25 μg/mL amphotericin B, 5 μg/mL vancomycin; 1:4: 2.5 μg/mL gentamicin, 0.62 μg/mL amphotericin B, 2.5 μg/mL vancomycin.

Table 2
Evaluation of growth temperatures and monitoring methods: use of different monitoring methods for following the SV assays, inoculated with different microorganisms and incubated at different temperatures.

	Temperature (°C) (positive SV/total SV)	Monitored by	Days after inoculation						
			7 days	9 days	12 days	17 days	19 days	24 days	29 days
Rickettsia conorii	28 °C (4/4)	Gimenez[a]	0–5 (3–4)	0–5 (3–4)	5–20 (6–7)	25–40 (8–9)	25–40 (8–9)	25–40 (8–9)	25–40 (8–9)
		IFA	ND[b]	+	ND	+	ND	+	+
		PCR	+	+	+	+	+	+	+
	32 °C (4/4)	Gimenez	0–5 (7)	0–5 (7)	25–45 (8–9)	25–45 (8–9)	25–45 (8–9)	25–45 (8–9)	25–45 (8–9)
		IFA	ND	+	ND	+	ND	+	+
		PCR	+	+	+	+	+	+	+
	37 °C (4/4)	Gimenez	0–5 (3–4)	0–5 (3–4)	5–20 (6–7)	25–40 (8–9)	25–40 (8–9)	25–40 (8–9)	25–40 (8–9)
		IFA	ND	+	ND	+	ND	+	+
		PCR	+	+	+	+	+	+	+
R. massiliae-Bar29	28 °C (1/4)	Gimenez	–	0–5 (3)	0–5 (3)	≥ 15 (7–8)	≥ 15 (7–8)	≥ 15 (7–8)	[–][c]
		IFA	ND	ND	ND	ND	ND	+	[ND][c]
		PCR	+	+	+	+	+	+	[–][c]
	32 °C (1/4)	Gimenez	–	–	–	–	0–5 (3)	5–15 (4)	5–15 (4)
		IFA	ND	ND	ND	ND	ND	ND	+
		PCR	–	–	+	+	+	+	+
	37 °C (0/4)	Gimenez	–	–	–	–	–	–	–
		IFA	ND	–	ND	–	ND	–	–
		PCR	–	–	–	–	–	–	–

[a] Minimum and maximum of free bacteria observed by FOV (Score value of Vero cell infection, from value = 0 [no microorganisms into Vero cell] to value = 10 [Vero cells full of rickettsia].
[b] ND: not done.
[c] The only positive SV were used for IFA detection on the 24th day, the other SV were monitored until the 29th day obtaining negative results.

for the first time by real-time PCR into a SV incubated at 28 °C on the 7th day when no microorganisms were observed by Gimenez staining. Afterwards, it was detected by Gimenez staining on the 9th day into the same SV. At that moment, Vero cells were 30–40% full of *R. massiliae*-Bar29 and 0–5 microorganisms were observed for field of view free in the supernatant. At 32 °C, *R. massiliae*-Bar29 was detected for the first time after 12 days of incubation by real-time PCR. In this SV, microorganisms were not seen by Gimenez staining until the 19th day. At that moment, 0–5 *R. massiliae*-Bar29 for field of view were observed free in the supernatant. IFA assay of the positive SV incubated at 32 °C, which was performed at the end of follow-up (29 days of incubation), was positive. IFA assay of the positive SV incubated at 28 °C, which was performed on the 24th day, was positive. IFA assay performed on the other ten SV were negative. More detailed information is shown in Table 2.

3.2. Evaluation of antibiotics

Data obtained is shown in Table 3. In those SVs inoculated with a biopsy or a tick, the highest concentration of antibiotics and the 3:4 dilution avoided the contamination. However, contamination was observed in those SV incubated with 1:4 and 1:2 dilutions, after 1–2 weeks and 4 weeks respectively.

After 1 week of incubation, *R. conorii* was detected, by both Gimenez staining and IFA assay, into SVs in which either 1:2 or 1:4 dilutions of antibiotics had been added. After 2 weeks of incubation, *R. conorii* was detected, by both methods, into all SVs of the experiment. *R. massiliae*-Bar29 was detected since 3rd week, at 3:4, 1:2 and 1:4 dilutions of antibiotics, by both Gimenez staining and IFA assay. After 4 week of incubation, *R. massiliae*-Bar29 was detected at all concentrations of antibiotics, by both methods. Concentrations of antibiotics corresponding to the 3:4 dilution were chosen as the best ones of the experiment. They are as follows: 7.5 μg/mL gentamicin, 1.88 μg/mL amphotericin B, 7.5 μg/mL vancomycin.

4. Discussion

During the last years, the number of new rickettsial strains and species has been increasing due to the improvement of molecular techniques. This fact has brought about a challenge for the properly identification of species. One example can be clinical cases related to Mediterranean spotted fever (MSF) whose etiology is associated with not only *R. conorii* but also *R. massiliae*-Bar29 (Cardeñosa et al., 2003; Parola et al., 2013; Vitale et al., 2006). Since the most used diagnosis method, indirect immunofluorescence assay (IFA), does not allows to accurately distinguish the species; other approach should be carried out. The aim of this study was the analysis of different features of Shell-vials (SV) culture assay in order to improve the detection and isolation of these microorganisms.

Classically, SV has been monitoring by the indirect immunofluorescence assay (Cardeñosa et al., 2000; Espejo-Arenas and Raoult, 1989; Marrero and Raoult, 1989; Péter et al., 1990) or the Gimenez staining (Babalis et al., 1994; Beati et al., 1996; Milhano et al., 2010; Psaroulaki et al., 2005). In our study, we analyzed the usefulness of the real-time PCR as a monitoring method. Our data show that slower and more difficult growth, more useful are the molecular

Table 3
Results of evaluation of antibiotics: follow-up of SV cultures inoculated with *R. conorii, R. massiliae-Bar29*, biopsy or tick and incubated with different antibiotic concentrations.

		Antibiotic concentration[a]															
		T				3:4				1:2				1:4			
		RC[b]	B29	b	t	RC	B29	b	t	RC	B29	b	t	RC	B29	b	t
1st week	Gimenez	–	–	–	–	–	–	–	–	+	–	–	–	+	–	C[c]	–
	IFA	ND[d]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND		ND
2nd week	Gimenez	+	–	–	–	+	–	–	–	+	–	–	–	+	–		C
	IFA	+	–	–	–	+	–	–	–	+	–	–	–	+	–		
3rd week	Gimenez	+	–	–	–	+	+	–	–	+	+	–	–	+	+		
	IFA	+	–	–	–	+	+	–	–	+	+	–	–	+	+		
4th week	Gimenez	+	+	–	–	+	+	–	–	+	+	C	C	+	+		
	IFA	+	+	–	–	+	+	–	–	+	+			+	+		

[a] Those concentrations shown in Table 1.
[b] RC: *Rickettsia conorii*; B29: *R. massiliae-Bar29*; b: biopsy; t: tick.
[c] Contamination.
[d] Not done.

methods. In fact, the slower growth was observed in SV inoculated with *R. massiliae*-Bar29 incubated at 32 °C. In this case, *R. massiliae*-Bar29 was detected a week earlier by real-time PCR than by Gimenez staining. At 28 °C, which seemed a more optimal temperature for *R. massiliae*-Bar29 growth, it was detected two days earlier by real-time PCR. On the contrary, when microorganism growth was easy and fast, IFA and Gimenez staining were as useful as real-time PCR. This fact was observed in *R. conorii* SV cultures. Although we considered our Gimenez staining as positive, it should be taking into account that the detection of microorganisms by this method in our study consisted of an exhaustive and independent search by two researchers. This in-depth search would be impossible to be performed routinely. As a consequence, samples showing very low amount of microorganisms by Gimenez staining in our study may have been probably considered negative if no other test had been performed. As a consequence, a routinely monitoring with a real-time PCR can allow not to discard samples in which microorganisms could be detected and observed later.

Different growth temperatures were tested. It was observed a rapid growth of *R. conorii* at all of them although the inoculum was so low. This fact agrees with previous studies in which *R. conorii* has been detected after 2–14 days of incubation (Cardeñosa et al., 2000; Espejo-Arenas and Raoult, 1989; Psaroulaki et al., 2005). *R. conorii* has been usually cultured at 32 °C (Psaroulaki et al., 2005; Renesto et al., 2008) or at 37 °C (Cardeñosa et al., 2000; Marrero and Raoult, 1989; Péter et al., 1990; Rovery et al., 2005) and fewer publications describe its growth at 28 °C (Uchiyama, 2005). Different kind of culture cells had been used in those studies, thus, our objective was to compare *R. conorii* growth at different temperatures using the same inoculum and cell type. In spite of growth was not quantified, which may be a study limitation, our data point toward 32 °C is a better incubation temperature than 28 °C or 37 °C

R. massiliae-Bar29 growth was observed neither at all temperatures nor in all shell-vials of each temperature. Some factors could explain our observations: microorganisms may have not been enough viable after a freezing period, they had slower growth rate or there was a too low amount of microorganism in the diluted inoculum. Taking into account *R. massiliae*-Bar29 had been cultured before the experiment; the inoculum used was from a viable culture. On the other hand, as the comparison between both species was not the objective, the design of the study does not allow to determine if *R. massiliae*-Bar29 growth rate is slower than that of *R. conorii*. However, a previous study did talk about a different growth pattern between both species, which may explain the failure to isolate *R. massiliae*-Bar29 from patients (Cardeñosa et al., 2000). Whether *R. massiliae*-Bar29 had a slower growth rate, or the inoculum contained low amount of microorganisms, or *R. massiliae*-Bar29 could not be enough viable, we can find these factors in a real sample. Therefore, increasing the incubation periods of shell-vials, even after approximately three weeks, as well as increasing the number of shell-vials for sample could be useful for avoiding false negatives. Long incubation periods for *R. massiliae* culture, even longer than 4 weeks, have been described (Milhano et al., 2010; Eremeeva et al., 2006). They could be difficult to keep in a routine clinical laboratory. However, the application of a high sensitive real-time PCR has been demonstrated as a useful method for earlier detection and identification of the microorganism. Likewise, if a laboratory is trying to isolate this bacterium, it should be aware of the possibility that longer incubation periods could be required.

In our study, data suggests that *R. massiliae*-Bar29 may grow better at 28 °C than at 32 °C. This fact agrees with Eremeeva et al. (2006) who pointed to incubation temperatures or culture cells could influence the growth rate of *R. massiliae*. In fact, their study showed that *R. massiliae* growth was improved when temperature was lowered to 28 °C. Likewise, although the proportion was not enough higher, Milhano et al. (2010) obtained one more *R. massiliae* at 28 °C than at 32 °C.

Finally, antibiotic concentrations were tested for the isolation of these microorganisms from the most frequent non-sterile samples such as biopsies and ticks. There were not much information and it was non-standardized. After a revision of different publications (Horta et al., 2006; Labruna et al., 2004; Melles et al., 1999; Paddock et al., 2004, 2006), gentamicin, amphotericin B, and vancomycin were chosen due to all together cover most microorganisms' spectrum. The same growth temperature was chosen for testing both species because their clinical symptoms can be similar, both can be found in the same vectors and, thus, it would be necessary a method that includes both species at the same time. In order to be sure that antibiotics would not avoid the growth of these species in no situation, an incubation temperature of 32 °C was chosen because it had been the worst condition for *R. massiliae*-Bar29 growth. Appropriate antibiotic concentrations were found for both *R. conorii* and *R. massiliae*-Bar29. In addition, it seemed not to affect too much their growth compared with their previous observed growth without antibiotics.

5. Conclusions

To sum up, real-time PCR can be a useful method to monitor SV assays, particularly when different factors (low inoculum, slow growth rate, non-appropriate culture conditions. . .) can have a bad effect on the culture. In addition, when a MSF diagnosis is

suspected, it could be advantageous to incubated SV at both 28 °C and 32 °C because of our observation of *R. massiliae*-Bar29 growth. Finally, as antibiotics useful for the isolation of *R. conorii* and *R. massiliae*-Bar29 have been described, non-sterile samples, in which the detection efficiency is suspected to be higher, can be used.

Acknowledgements

This study was supported by Instituto de Salud Carlos III grant FIS12/02308, Fundació Parc Taulí grant CIR 2012/044 and postdoctoral fellowship of the Spanish Society of Infectious Diseases and Clinical Microbiology – SEIMC.

We would like to thank Jessica Tijero for her technical support.

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