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