

Reorganization of actin cytoskeleton in L929 cells infected with *Coxiella burnetii* strains isolated from placenta and foetal brain of sheep (Sardinia, Italy)

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Keywords

Actin cytoskeleton,
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Q Fever.

Summary

Coxiella burnetii, the etiological agent of Q Fever, is a zoonotic pathogen distributed worldwide. It has been reported that virulent strains of *C. burnetii* are poorly internalized by monocytes compared to avirulent variants. Virulence is also associated to the formation of pseudopodal extensions and transient reorganization of filamentous actin. In this article, we investigated the ability of 2 *Coxiella* strains isolated from ovine aborted samples to induce reorganization of the actin cytoskeleton in mouse fibroblast cells. Cells were exposed for 24 and 48 hours to ovine placenta and foetal brain tissue homogenates and then analysed by polymerase chain reaction (PCR) in order to detect *Coxiella* infection. The formation of pseudopodal extensions, the polarized distribution of F-actin, and the involvement of *C. burnetii* strain in cytoskeleton reorganization have been assessed using a laser scanning confocal fluorescence microscope. Results indicate that similarly to the virulent reference strain, strains of *C. burnetii* isolated from foetal brain induced morphological changes – modification in F-actin distribution and in the localization of bacteria. By contrast, *C. burnetii* strain isolated from ovine placenta did not induce any significant change in L929 cell morphology. In conclusion, both *C. burnetii* strains isolated from ovine placenta and foetal brain were pathogenic causing ovine abortion, but *in vitro* the *C. burnetii* strain isolated from brain only was able to induce F-actin reorganization in L929 infected cells.

Riorganizzazione dell'actina del citoscheletro in cellule L929 infettate con ceppi di *Coxiella burnetii* isolati da placenta di pecora e cervello fetale ovino (Sardegna, Italia)

Parole chiave

Actina del citoscheletro,
Ceppi di *Coxiella burnetii*,
Fagocitosi,
Febbre Q,
Microscopio confocale a
fluorescenza.

Riassunto

Coxiella burnetii, l'agente eziologico della Febbre Q, è un patogeno zoonotico presente in tutto il mondo. È stato riportato che l'internalizzazione dei ceppi virulenti è meno efficiente rispetto ai ceppi avirulenti. La virulenza di un ceppo è stata anche associata alla capacità di formare pseudopodi e di riorganizzare in modo transitorio l'actina del citoscheletro cellulare. In questo lavoro, è stata studiata la capacità di due ceppi di *Coxiella* isolati da aborti ovinii di indurre la riorganizzazione dell'actina del citoscheletro in cellule di fibroblasto di topo. Le cellule sono state infettate per 24 e 48 ore con omogenati di placenta e cervello e sottoposte a PCR per valutare l'infezione di *Coxiella*. Inoltre, tramite microscopio confocale a fluorescenza è stata valutata l'abilità di alcuni ceppi di *Coxiella burnetii* di formare pseudopodi, di localizzare l'actina F polarizzata e riorganizzare il citoscheletro cellulare. I risultati indicano che il ceppo di *C. burnetii*, similmente al ceppo virulento di riferimento, isolato dal campione di cervello di feto ovino induceva modificazioni morfologiche (modificazioni nella distribuzione dell'actina e nella localizzazione batterica). Al contrario, il ceppo di *C. burnetii* isolato dalla placenta ovina non ha indotto alcun cambiamento significativo nelle cellule L929. In conclusione, entrambi i ceppi di *C. burnetii* isolati dalla placenta ovina e dal cervello fetale ovino sono stati in grado di causare aborti ovinii ma, in laboratorio, il ceppo isolato dal cervello fetale è stato l'unico capace di riorganizzare l'actina F nelle cellule L929 infettate.

Introduction

Coxiella burnetii (*C. burnetii*) is a gram-negative obligate intracellular acidophile bacterium that is highly adapted to live within the eukaryotic phagolysosome (Maurin Raoult 1999, Mege *et al.* 1997). It is the etiological agent of Q Fever, a pathology characterized by acute and chronic courses. Recently, *C. burnetii* has been propagated in axenic media, thus it is not considered an obligatory intracellular bacterium anymore (Omsland *et al.* 2011). Infections with this zoonotic pathogen occur worldwide. In human, the main route of infection is inhalation of contaminated aerosol or dust containing bacteria shed by infected animals through milk, faeces, vaginal or placenta secretions during animal birthing (Arricau-Bouvery and Rodolakis 2005, Arricau-Bouvery *et al.* 2006, Rodolakis 2009). Both the high concentration of infectious organisms released at parturition and the remarkable resistance of the organism to environmental extremes underlie the heavy environmental contamination associated with parturient livestock. Concerning its pathogenicity, it seems that it varies according to country, region, climate, type of animal, system of management and other circumstances, including virulence of the agent (Agerholm 2013).

Different virulence levels of infections have been observed but it is still not clear whether disease severity is the result of a variability in bacterial virulence factors or depends on the immunological background of the host (Arricau-Bouvery *et al.* 2006).

The antigenic variation, also known as phase variation, is one of the most important aspects of *C. burnetii*. In Phase I, organisms isolated from acutely infected animals, arthropods or humans express a wild virulent form, characterised by smooth full-length lipopolysaccharide (LPS).

Avirulent Phase II bacteria show a truncated, rough LPS molecule. Differences in surface protein composition, surface charge, and cell density may also be detected. Transition from virulent Phase I to an avirulent Phase II requires several serial passages in embryonated eggs or cell cultures (Hotta *et al.* 2002, Marrie 2000).

Coxiella burnetii enters monocytes/macrophages, the only known target cells, by phagocytosis that differs in Phase I and Phase II cells (Angelakis and Raoult 2010). *Coxiella burnetii* virulence depends on its ability to enter macrophages and to escape from their microbicidal activity. Virulent strains increase their content in filamentous actin (F-actin) and induce its intense and transient rearrangement. As the phagocytosis of virulent *C. burnetii* is associated with the formation of pseudopodal extensions and polarized distribution of F-actin (Ghigo *et al.* 2002, Ghigo *et al.* 2009), we have characterized the isolates

by assessing F-actin localization in cytoskeleton reorganization. The objective of this paper is to determine the virulence of *C. burnetii* strains isolated from placenta and brain of sheep abortion in Sardinia, Italy, by comparison with a virulent reference strain of *C. burnetii*. For this purpose, we evaluated the effect of isolated strains on reorganization of host actin cytoskeleton during infection.

Materials and methods

Preparation of clinical samples, DNA isolation and PCR

Ovine placental and foetal brain tissue samples collected from 2 sheep aborted fetuses collected during a Q fever outbreak which occurred in Sardinia in 2012 were used to discriminate among *C. burnetii* isolates. Samples were washed with sterile phosphate-buffered saline (PBS, 0.1M phosphate, 0.33 M NaCl), pH 7.2 containing 1,000,000 units/ml of penicillin G sodium salt (Fluka-Sigma-Aldrich, St. Louis, Mi, USA), and 1,000,000 units/ml of streptomycin sulphate salt (Fluka-Sigma-Aldrich, St. Louis, Mi, USA). They were then digested for 3 hours at 37°C with 0.6% and 2% trypsin solutions (Difco, Detroit, Mi, USA), respectively. Digested tissues were filtered with an aseptic gauze and centrifuged at 6,000 x g for 10 min. DNA extraction was then performed using a DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed to evaluate the presence of *C. burnetii*, using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The assay amplified specifically a 257 bps region within the superoxide dismutase (SOD) gene (F: 5'-ACTCAACGCACTGGAACCGC-3'; R: 5'-TAGCTGAAGCCAATTCGCC-3') of *C. burnetii* (Stein and Raoult 1992). Positive control DNAs were also extracted from *C. burnetii* (Nine mile/I/ EP1). Water samples were included in all amplifications as a negative control; PCR products were resolved on a 1.5% agarose gel in TAE buffer (0.04 M Tris/acetate, 0.001 M EDTA). After electrophoresis at 100 V for 60 min, gels were stained with ethidium bromide and examined over UV light.

Infection of L929 cells with *C. burnetii* from reference strain, placenta and brain homogenates

Coxiella burnetii (Nine Mile/I/EP 1) and pellet of digested tissue samples were propagated in mouse fibroblast cells (L-929; ECACC n°85011425) at a bacterium-to-cell ratio of 200:1 as described by Meconi and colleagues (Meconi *et al.*, 1998) and cultured in DMEM (Dulbecco's modified eagle

medium, Gibco-Life Technologies, Eragny, France) supplemented with 4% foetal bovine serum and cultivated at 37°C in an atmosphere containing 5% CO₂ into 25 cm² tissue culture flasks containing a semi-confluent monolayer of L929 cells at an initial density of 4x10⁵ cells/ml. The culture medium was replaced daily with fresh medium. The vitality of organisms was assessed by observing the characteristic motility of bacteria into vacuoles per cell with an inverted optic microscope (40X) after 24 and 48 hours from infection. Moreover, PCR was performed in order to support the culture infection. All other methods pertaining to the cell culture have been implemented in accordance with established procedures. All the propagative methods and those related to the manipulation of the reference strain, placentas, and foetal brain tissues from domestic ruminant were performed under Biosafety level 3 (BSL3) conditions.

Laser scanning confocal fluorescence microscopy analysis

Cells cultured for 24 and 48 hours were displaced into chamber slide, fixed with 4% paraformaldehyde (pH 7.3-7.4), and incubated over night at room temperature. Cells were then permeabilized with 0.1% saponin (Fluka-Sigma-Aldrich, St. Louis, Mi, USA) in phosphate buffered saline (PBS) containing 10% horse serum (Invitrogen, Carlsbad, CA, USA) for 30 min at room temperature. Cells were rinsed with PBS 3 times. In order to detect a possible reorganization of F-actin induced by *C. burnetii*, L929 cells were incubated with a mix of 10 U/ml Alex fluor 555 phalloidin for F-actin intracellular distribution staining and 0,1mg/ml of DAPI (Molecular Probes, Eugen, Oregon, USA) for nucleus staining at 37°C for 30 minutes in the dark. In addition, for bacteria localization, cells were incubated with 500 µl of anti-*Coxiella* positive human serum (QGP-110927, Fuller Laboratories, Fullerton, CA, USA) at 37°C for 30 minutes. *Coxiella burnetii* was

revealed by using 100 µl of fluorescein-coniugated goat anti-human immunoglobulin serum (KPL). Thereafter, cells were washed, mounted with Mowiol (Calbiochem, San Diego, CA, USA) and stored at 4°C in the dark. Samples were then examined with a laser scanning confocal fluorescence microscope (LAS AF, Leica Microsystem-Application Suite Advanced Fluorescence).

Results

After 24 hours from infection, control cells were vigorously grown as colonies and were confluent (Figure 1a) conversely, about 80% of cells infected with *C. burnetii* Nine Mile I showed a cytopathic effect characterized by increased size with membrane extensions and polarised protrusions. The growth was inhibited and numerous cells were detached from the monolayer and floating in the medium (Figure 1b). Cells infected with *C. burnetii* strains isolated from placenta and brain tissues did not show morphological modifications after 24 hours. After 48 hours from infection, cells infected with *C. burnetii* Nine Mile I and with *C. burnetii* strain isolated from foetal brain exhibited alterations in morphology such as cell spreading, membrane extensions, polarized filopodia, and lamellipodium protrusions (Figure 1c). *C. burnetii* strain isolated from sheep placenta did not show any morphological cell alteration at 48 hours post-infection.

After 24 hours from infection, fluorescently-labeled phalloidin for labelling and localization of F-actin, produced an homogenous and peripheral ring of F-actin in control cells (Figure 2a). By contrast, staining of cells infected with *C. burnetii* Nine Mile I showed F-actin inside the protrusion and in the areas away from cell deformation (Figure 2b). Cells infected with *C. burnetii* strains isolated from placenta and brain tissues did not show pseudopodal extensions or polarized distribution of F-actin (Figure 2c and Figure 2d). After 48 hours, 80% of L929 infected with



Figure 1. Morphological features of L929 observed in the living state with an inverted optic microscope (40X): control cells (a) and cells infected with *C. burnetii* Nine Mile I (b) 24 hours after infection; cells infected with *C. burnetii* strain isolated from foetal brain 48 hours after infection (c).

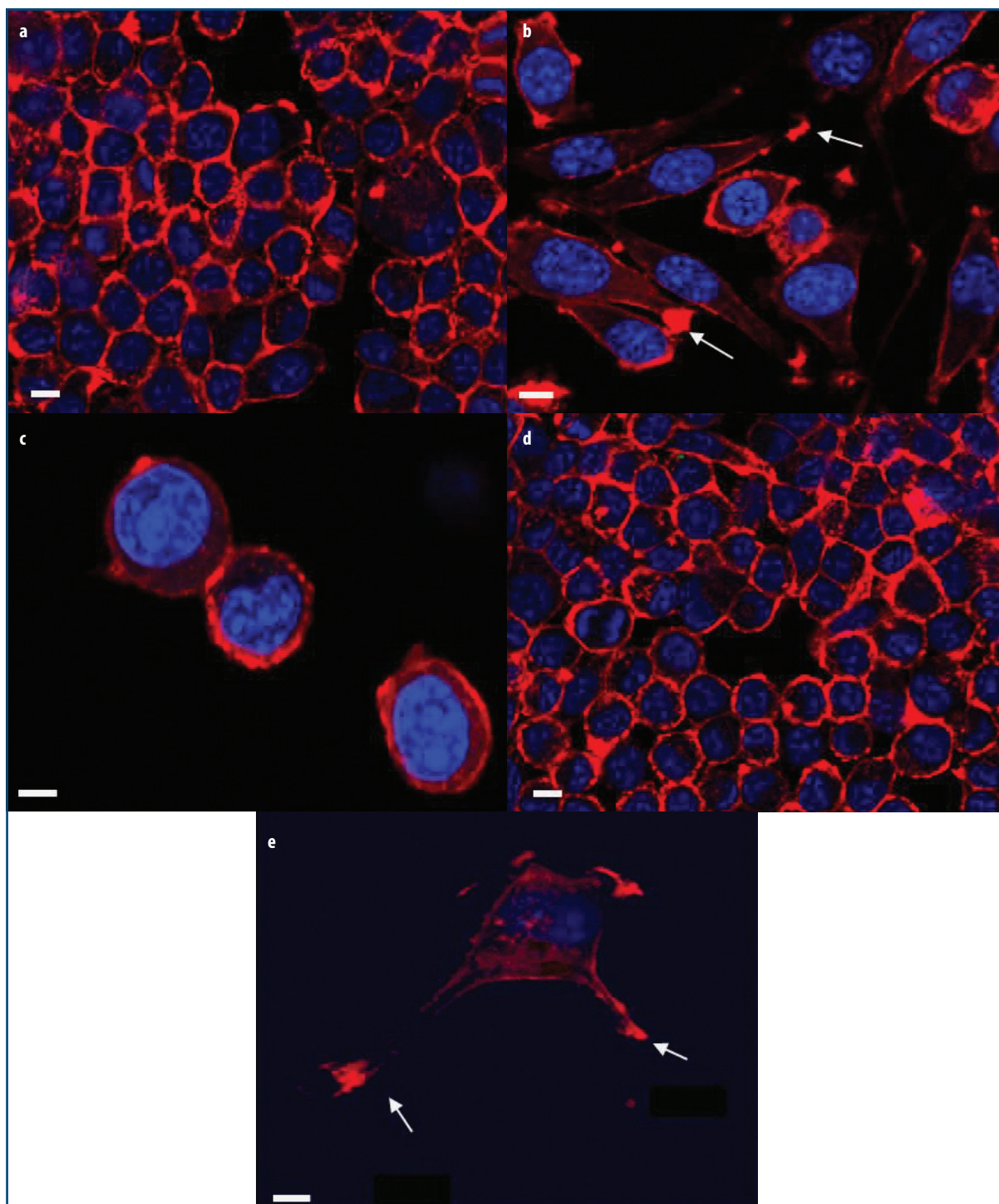


Figure 2. Reorganization of F-actin induced by *C. burnetii* after incubation with DAPI (in blue), for nucleus staining and Alex fluor 555 phalloidin (in red), for intracellular distribution of F-actin staining: control L929 cells (a). L929 cells infected with *C. burnetii* Nine Mile I (b). L929 infected with *C. burnetii* strain isolated from placenta after 24 hours (c). L929 cells infected with *C. burnetii* strain isolated from foetal brain after 24 and 48 hours from infection, respectively (d and e). The protrusions are pointed out by arrows. Objective HCX PL APO lambda blue 40.0 x 1.15 OIL UV, Emission band with PMT 2: begin end 513 nm-546nm. Bars, 10 µm.

C. burnetii strain isolated from foetal brain showed an alteration in F-actin distribution that resulted inside the protrusion and around the cells (Figure 2e).

Coxiella burnetii localization was observed by double fluorescence after 24 and 48 hours from infection.

The localization of bacteria closely apposed to the protrusion in cells infected with *C. burnetii* Nine Mile I after 24 hours is shown in Figure 3a. In L929 cells infected with *C. burnetii* strain isolated from sheep placenta, the bacteria occupied a dominant portion of the cytoplasmic space of the cell (Figure 3b). L929

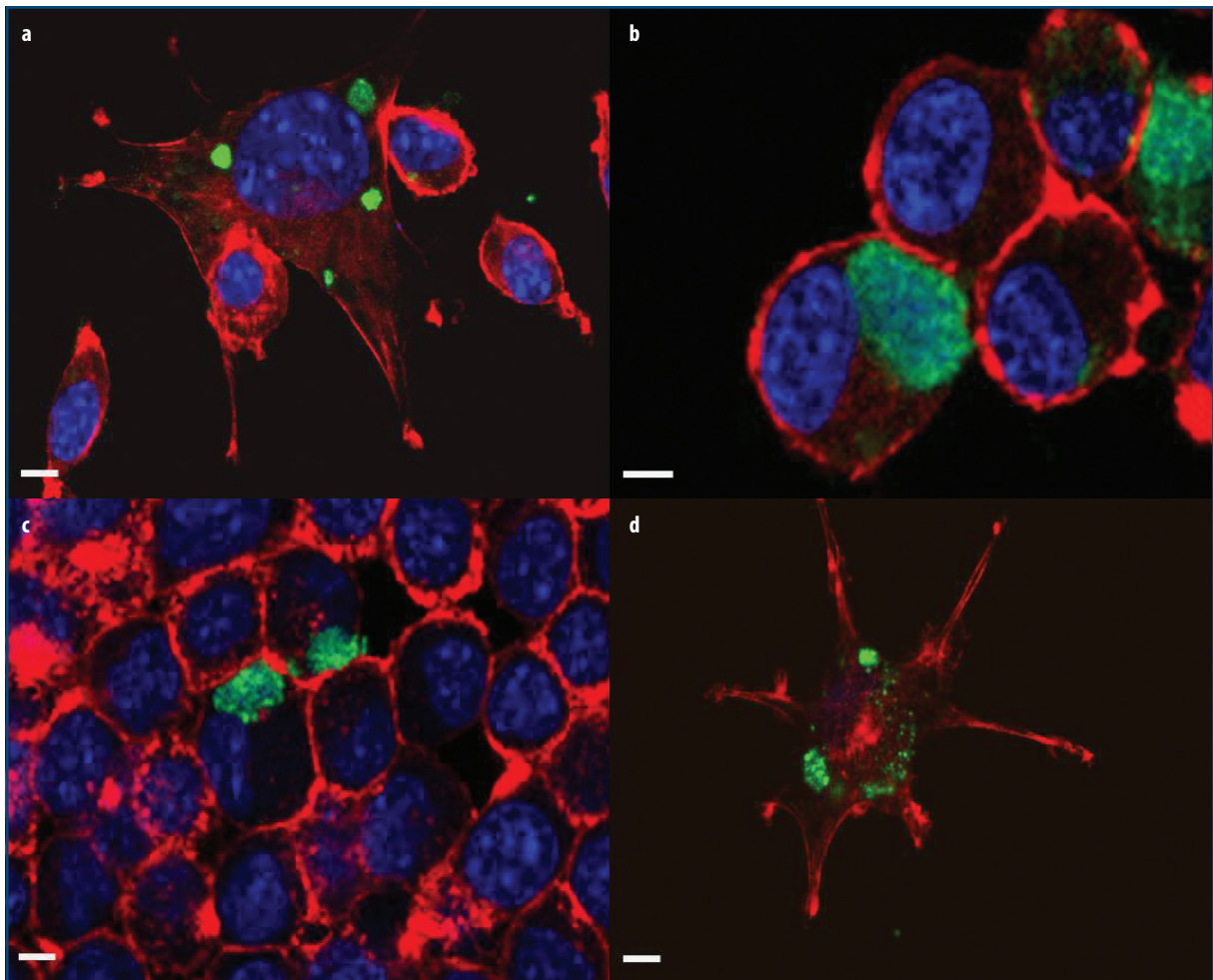


Figure 3. Localization of *C. burnetii* in L929 cells infected with: *C. burnetii* Nine Mile I (a); *C. burnetii* strain isolated from sheep placenta (b); *C. burnetii* strain isolated from foetal brain 24 and 48 hours (c and d) from infection. Nucleus, intracellular distribution of F-actin and bacteria were labeled with DAPI, Alex fluor 555 phalloidin and FITC, respectively. Objective HCX PL APO lambda blue 40.0 x 1.15 OIL UV, Emission band with PMT 2: begin end 511 nm-545nm. Bars, 10µm.

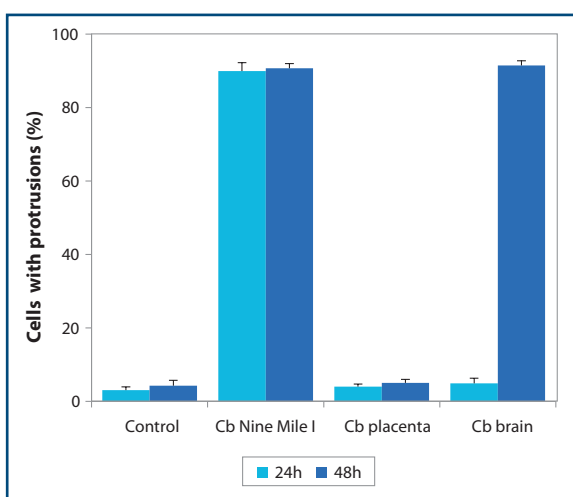


Figure 4. Percentage of L929 cells showing protrusions 24 and 48 hours after infection with incubated with *C. burnetii* reference strain (Cb Nine Mile I), *C. burnetii* strain isolated from sheep placenta (Cb placenta) and *C. burnetii* strain isolated from foetal brain (Cb brain). Controls were represented by L929 non-infected cells. The histogram represents the mean \pm SD of 4 experiments.

cells infected with *C. burnetii* strain isolated from brain showed a low fluorescence after 24 hours from infection (Figure 3c). After 48 hours, the same cells showed cytoplasmic extensions and *Coxiella* presence near F-actin protrusion, as shown in Figure 3d.

The correlation between *C. burnetii*-infected cells showing membrane protrusion and the time of infection is represented in Figure 4. *Coxiella burnetii* Nine Mile I induced F-actin reorganization in 90 ± 3 % of L929 cells both at 24 and 48 hours. No F-actin polymerization was observed after 24 or 48 hours in L 929 cells infected with *C. burnetii* strain isolated from placenta. An increase in the level of F-actin reorganization occurred only after 48 hours in cells infected with *C. burnetii* strain isolated from brain.

Discussion

This is the first report of detection, identification, and isolation of *C. burnetii* from ovine abortion samples

in Sardinia, Italy. Even though the traditional technique used for isolation of the bacterium is the shell vial centrifugation assay (Gouriet *et al.* 2005), the culture method used in the present study allowed the isolation of *C. burnetii* from ovine samples due to their high concentration of bacteria, which facilitated the isolation.

In vitro, *C. burnetii* infects several cultured cell lines, such as P338D1 mouse macrophage-like cells (Akporiaye *et al.* 1983), L929 fibroblast cells (Baca *et al.* 1985), and Vero cells (Maurin and Raoult 1999). Differences in the cellular response to infection have been observed (Graham *et al.* 2013).

The bacterium resides in an acidic parasitophorous vacuole (PV) with late endosome-lysosome characteristics (Aguilera *et al.* 2009). Recently, PV has also been shown to interact with the autophagic pathway, acquiring autophagosomal features (Beron *et al.* 2002, Gutierrez *et al.* 2005, Romano *et al.* 2007). An important aspect worth considering is the virulent *Coxiella's* ability to enter into macrophages and to escape from their microbicidal activity (Honstetter *et al.* 2004). Indeed, virulent organisms are poorly internalized and survive in monocytes, whereas avirulent variants are efficiently phagocytosed but are eliminated (Meconi *et al.* 2001).

The virulence and pathogenic mechanisms of *C. burnetii* are not clearly understood but it is generally accepted that the bacterial LPS is important in the pathogenesis of Q fever in humans and animals and it is the only defined virulence factor of *C. burnetii* (Woldehiwet 2004).

Eukaryotic host cell cytoskeleton (actin filaments, microtubules, and intermediate filaments) are a common target of molecular interactions for intracellular microbial pathogens (Bhavsar *et al.* 2007). The actin cytoskeleton is differentially modulated to support bacterial internalization (Meconi *et al.* 1998). It has been shown that virulent *C. burnetii* affects F-actin reorganization in THP-1 human monocyte cells and that changes in cell morphology can be associated with the mobilization of actin cytoskeleton and consequent formation of pseudopodal extensions and polarized distribution of F-actin (Meconi *et al.* 1998, Meconi *et al.* 2001). Only virulent organisms induce cell protrusions rich in F-actin in monocytes, suggesting that actin cytoskeleton is involved in the control of *C. burnetii* phagocytosis (Meconi *et al.* 1998).

The results of this study suggest that *C. burnetii* strain isolated from foetal brain stimulates the reorganization of actin cytoskeleton inside cell protrusions also in L929 non-macrophage-like cells.

Coxiella burnetii entered slowly into fibroblasts and only after 48 hours from infection cells showed membrane protrusions and polarized projections and bacteria resulted closely apposed to the protrusion of F-actin into the characteristic PV. Unlike other intracellular bacteria that use mechanisms to evade endocytic pathways, *C. burnetii* has a unique intracellular life cycle. After internalization into a host cell, *C. burnetii* establishes the PV that eventually fuses with compartments of the lysosomal network and expands to occupy the majority of the cytoplasmic space within the infected cells (Graham *et al.* 2013, Heinzen *et al.* 1996). This phenomenon is specifically related with F-actin that not only is recruited to but is also involved in the formation of the typical PV (Aguilera *et al.* 2009).

By contrast, *C. burnetii* strain isolated from ovine placenta did not induce any significant change in cell morphology after 24 or 48 hours from infection. Moreover, F-actin resulted as a homogeneous and peripheral ring around L929 cells demonstrating that bacteria belong to an avirulent strain. Avirulent organism did not stimulate the activation of Lyn and Hck, 2 src-related protein tyrosine kinases that lead to actin cytoskeleton reorganization involved in the impairment of phagocytosis of virulent *C. burnetii* (Meconi *et al.* 2001, Thomas and Brugge 1997).

In Italy, Q fever has been detected in Northern and Southern Italy; more specifically it has been recorded in both Sicilia and Sardinia in sheep, goats, cattle, and dogs by seroprevalence and PCR analysis (Baldelliet *et al.* 1992, Cabassiet *et al.* 2006, Capuano *et al.* 2004, Martini *et al.* 1994, Masala *et al.* 2004, Torina and Caracappa 2006). Both human and animal *C. burnetii* infections are under-diagnosed and under-reported. The diagnosis of Q fever is laboratory-based and requires expensive and elaborate methods and well-trained personnel to establish an unequivocal diagnosis. Since it requires biosafety laboratory level 3 conditions, it is rarely performed for routine diagnosis in veterinary medicine and restricted to laboratories specialized in cell-culture technique (Fournier *et al.* 1998). Further studies on aborted ovine fetuses are necessary for improving our knowledge about the virulence and pathogenicity of *Coxiella* strains, which causes ovine abortion in Sardinia.

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