Molecular detection of Coxiella burnetii *using an alternative loop-mediated isothermal amplification assay (LAMP)*

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Q fever.

Summary
Q fever, caused by *Coxiella burnetii*, is a worldwide zoonosis with important consequences for human and animal health. In livestock, the diagnosis, using direct and indirect techniques, is challenging even if to tackle coxiellosis in domesticated animals a rapid diagnosis is crucial. In the recent years, new molecular methods have been developed to overcome these issues. Several polymerase chain reaction (PCR) assays have been studied, but loop mediated isothermal amplification (LAMP) has not been fully developed. This new methodology is emerging due to simplicity and speed in diagnosis of microbial diseases. In this study, we design a new LAMP assay against *C. burnetii* targeting the *com1* gene as an actual alternative to conventional PCR. The assay was specific to *C. burnetii* reactive with sensitivity comparable to standard PCR. The application of the *com1* LAMP on 10 clinical samples from water buffalo, sheep, and goats, previously tested positive, confirmed the presence of *C. burnetii*. To our knowledge, this study is the first report of LAMP targeting *C. burnetii* in Europe and the results also suggest that it may be an useful and cost-effective tool for the clinical and epidemiological surveillance of Q Fever.

Sviluppo di un saggio alternativo di Amplificazione isotermica mediata da loop (LAMP) per la diagnosi di Coxiella burnetii

Parole chiave
Aborti infettivi,
Coxiella burnetii,
Febbre Q,
LAMP.

Riassunto
Coxiella burnetii, agente eziologico della Febbre Q è una zoonosi ubiquitaria causata da un batterio Gram negativo, pleomorfo, appartenente alla suddivisione dei γ-Proteobacteria. Per l'elevata resistenza e il grado di infettività nei confronti dell'uomo viene annoverato come potenziale agente di bioterrorismo. Ad ampia diffusione ambientale, il microrganismo può infettare una grande varietà di ospiti tra cui diverse specie di mammiferi domestici e selvatici. I ruminanti infetti, specie se giovani, manifestano sintomatologia clinica a carico della sfera riproduttiva con aborti tardivi e natimortalità rappresentando un pericoloso serbatoio di Febbre Q anche per l'uomo. L'inalazione di polveri contaminate e la manipolazione di organi infetti, costituiscono la più comune via di contagio. Una diagnosi rapida nei principali serbatoi animali della malattia resta una pratica fondamentale anche per ridurre i casi di Febbre Q nell'uomo. Le tecniche di diagnosi molecolare hanno permesso, negli ultimi anni, lo sviluppo di metodiche alternative alla PCR convenzionale. Tra queste, l'Amplificazione isotermica mediata da loop (LAMP) permette di evidenziare rapidamente la presenza di numerosi microorganismi patogeni utilizzando apparecchiature poco costose. Il lavoro ha avuto l'obiettivo di descrivere un nuovo saggio LAMP, disegnato sul gene *com1* presente in singola copia all'interno del genoma di *C. burnetii*, per la diagnosi di Febbre Q. La metodica ha permesso di rilevare la presenza del batterio in 10 campioni clinici appartenenti a diverse specie di ruminanti domestici. Il nuovo saggio LAMP rappresenta un'alternativa utile per la diagnosi di Febbre Q su invogli fetali, sia in alternativa che in associazione ai metodi convenzionali, e in tutti i casi in cui si richieda una diagnosi rapida della malattia.

Introduction

The proteobacterium *Coxiella burnetii*, a Gram-negative, pleomorphic, obligate intracellular pathogen, is the causative agent of both Q fever in humans and coxiellosis in animals. *Coxiella burnetii* is also a cause of abortion and stillbirth in goats, sheep, cattle, dogs, and cats, although the infection is usually asymptomatic in non-pregnant animals.

Small domestic ruminants are the primary animal reservoirs of the infection, and they shed these bacteria in milk, urine, and feces (Angelakis and Raoult 2010). Infected amniotic fluid and placental materials also are the usual vehicles of *C. burnetii* infection, with more than 109 bacteria/g of placental tissue at the time of delivery (Maurin and Raoult 1999) that can be released into the environment, and transmitted by inhalation of aerosols. Transmission is also possible through ingestion of contaminated food (Angelakis and Raoult 2010).

Coxiella burnetii can survive in the environment for long periods of time because of the strong resistance of the organism to physical and chemical agents (Maurin and Raoult 1999). Furthermore, *C. burnetii* is listed as a bioterrorism agent by the Centers for Disease Control, because it has a low infectious dose, and infections occur primarily through inhalation of contaminated soil, dust, or animal waste (Dorko *et al.* 2004).

Q fever in humans is a self-limited febrile illness, with the most frequent clinical manifestations being headache, myalgia, arthralgia, and cough (Raoult 2012). However, severe clinical complications have been reported (Fenollar *et al.* 2004). Outbreaks occur most frequently in areas that have a warm and windy climate (Dorko *et al.* 2004). In several regions of Italy, serological and molecular investigations have confirmed the presence of *C. burnetii* in various samples from water buffaloes (*Bubalus bubalis*) and in the milk of domestic animals, including cattle, sheep, goats, and dogs (Capuano *et al.* 2004, Monno *et al.* 2009, Parisi *et al.* 2006, Perugini *et al.* 2009, Vicari *et al.* 2013). A Q fever outbreak in US soldiers on duty in Grottaglie (Apulia Region) occurred in 1945 (Feinstein *et al.* 1946). Interestingly enough, the exact prevalence of *C. burnetii* in livestock from the Apulia Region remains unknown, probably because many cases of Q fever go unrecorded or are subclinical.

Loop-mediated isothermal amplification (LAMP) is an increasingly used molecular diagnostic technique that has shown high sensitivity and specificity for the diagnosis of Q fever (Pan *et al.* 2013). LAMP was introduced in 2000 by Notomi and colleagues (Notomi *et al.* 2000); it employs a unique constant temperature, a DNA polymerase, and 4 or 6 different primers, which increase the specificity of the assay.

The strand-displacing *Bst* polymerase creates amplification products that are stem-loop DNA structures with inverted repeats of the target and cauliflower-like structures with multiple loops (Notomi *et al.* 2000). The LAMP technique is an alternative diagnostic tool to conventional PCR that is now routinely used for the detection of many human and animal infectious agents.

The aim of this study was to prove the effectiveness of this methodology in the diagnosis of coxiellosis by testing abortion materials from domestic ruminants. In addition, we also compared the LAMP technique with traditional PCR designed on the same gene target.

Materials and methods

Analysis of tissue samples

Ten specimens previously found positive for *C. burnetii* using conventional PCR according to Berri and colleagues (Berri *et al.* 2009), were tested to evaluate the diagnostic efficiency of LAMP for detecting the bacteria. The material consisted of abortive products from domestic ruminants (7 from goats, 2 from sheep, and 1 from water buffalo) located in 10 animal farms between Apulia and Basilicata Regions. Samples were collected between March 1995 and September 2010 and stored at -80°C. DNA was extracted following the protocol of the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany).

LAMP assay

To develop the LAMP assay, 4 novel primers, FIP, BIP, F3, and B3 (Table I) were designed using Primer Explorer Software[1].

The primers were based on the *com1* gene, which is present as 1 copy only in all known *C. burnetii* genomes (Zhang *et al.* 1997) and encodes a 27-kDa outer membrane protein of *C. burnetii* (Genbank accession no. AB004712.1). This protein is the first outer membrane-associated immune reactive protein found in both acute and chronic Q fever disease (Hendrix *et al.* 1993). The LAMP reaction mixture (final volume, 25 µL) contained the following: 10 µL of isothermal amplification buffer 1X (20 mM Tris-HCl, 10 mM $[NH_4]_2SO_4$, 50 mM KCl, 2 mM $MgSO_4$, 0.1% Tween 20, pH 8.8), 5.2 µL nuclease free water, 2.5 µL of dNTPs (1.5 mM), 1.6 µM each of the FIP and BIP primers, 0.3 µM each of the F3 and B3 primers, 2.5 µL of extracted DNA, and 1 µL (8 U) of *Bst* 2.0 Warm Start DNA Polymerase (New England

[1] Lamp Primer designing Software. PrimerExplorer. http://primerexplorer.jp/e/.

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Biolabs Inc., Ipswich, MA, USA). The reaction mixture was incubated in a heating block at 65°C for 45 minutes and, subsequently, at 80°C for 5 minutes to terminate the reaction. The LAMP reaction was performed using the heating block T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). Three DNAs, extracted from uninfected human, sheep, and bovine blood samples respectively, were randomly used as negative controls.

Table I. *LAMP primer set.*

Primer type	Positions	Sequence 5′-3′
FIP	715-738/670-688	TCCTTGTTTAGCGGCTGCTAATGA-GAACTGCCCATTTTTGGCG
BIP	744-765/795-814	TTATGCTTTCCACGACGCGCTG-CTGCGGTTTGAAGGGTGATT
F3	649-666	AACCTCCGCGTTGTCTTC
B3	835-856	CCATGTCTTTTTTGAGCTGAGC

Gene target *C. burnetii com1* gene codifying for 27-kDa outer membrane protein (AB004712.1).

Table II. *Samples tested by* com1 *LAMP.*

A			
Sample id	Type of sample	Animal species	*Com1* LAMP
5467	Placenta	Goat (*Capra hircus*)	Positive
5644	Placenta	Water buffalo (*Bubalus bubalis*)	Positive
3759	Placenta	Sheep (*Ovis aries*)	Positive
833	Placenta	Goat (*Capra hircus*)	Positive
440	Placenta	Goat (*Capra hircus*)	Positive
1892	Placenta	Sheep (*Ovis aries*)	Positive
1898	Placenta	Goat (*Capra hircus*)	Positive
830	Placenta	Goat (*Capra hircus*)	Positive
X023	Placenta	Goat (*Capra hircus*)	Positive
X024	Placenta	Goat (*Capra hircus*)	Positive
CHB1	Blood	Human (*Homo sapiens*)	Negative
COB2	Blood	Sheep (*Ovis aries*)	Negative
CCB1	Blood	Goat (*Capra hircus*)	Negative

B		
Classification	Name of pathogen	*Com1* LAMP
Members of the order Rickettsiales	*Rickettsia helvetica, Rickettsia aeschlimannii, Rickettsia conorii, Rickettsia felis, Rickettsia monacensis, Rickettsia prowazekii, Rickettsia siberica, Rickettsia slovaca, Rickettsia typhi, Orientia tsutsugamushi*	Negative
Potential bioterrorism agents	*Brucella abortus, Brucella canis, Brucella melitensis, Bacillus anthracis, Yersinia pestis, Burkholderia mallei, Burkholderia pseudomallei, Francisella tularensis*	Negative
Other common pathogenic microorganisms	*Bartonella henselae, Bartonella quintana, Brucella neotomae, Brucella ovis, Yersinia pseudotuberculosis, Staphylococcus aureus, Streptococcus equi, Escherichia coli* VTEC, *Chlamydophila abortus, Chlamydophila psittaci, Salmonella* spp., *Anaplasma phagocytophilum, Borrelia burgdorferi, Ehrlichia chaffeensis, Babesia ovis*	Negative

A = Clinical samples;
B = Laboratory strains for determining the specificity of LAMP assay.

Determination of specificity of the LAMP assay

The specificity of the *C. burnetii* LAMP assay was evaluated by testing 33 bacterial DNAs (Table II): 10 DNA samples from the family *Rickettsiaceae* and phylogenetic related pathogens, 8 DNA samples from potential bacterial agents of bioterrorism, and 15 other DNA of common pathogens. The NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used to determine the DNA concentrations of all the samples. DNA concentrations ranged from 88 to 120 ng/µL. The DNA of every sample was diluted using nuclease-free water (QIAGEN, Hilden, Germany) to achieve a final concentration of 80 ng/µL before testing.

Determination of sensitivity of the LAMP assay

To test analytic sensitivity, 10-fold serial dilutions of *C. burnetii* DNA (Nine Mile strain) from 100 ng to 100 fg were subjected to LAMP.

The LAMP products were analyzed directly using UV illumination after the addition of propidium iodide to each tube (1:10 dilution of 10 mg/mL stock solution) (Hill *et al.* 2008).

PCR assay

A novel PCR assay based on the same gene target of the LAMP was performed. The aim of this new PCR was to compare the sensitivity between the 2 molecular methods designed on the sequence of the *com1* gene. The PCR assay employed the LAMP 'outer' primers F3 and B3 and amplified a 208-bp fragment of the *C. burnetii com1* gene (Raele, unpublished data). This PCR assay was carried out in a 25 µL reaction mixture containing the following: 12.5 µL RED Taq Ready Mix 1x (Sigma-Aldrich, St. Louis, MO, USA), 9.5 µL nuclease free water, 0.5 µM each of F3 and B3 primers, and 2.5 µL of extracted DNA. The reaction mixture was first subjected to 94°C for 7 minutes and then 35 cycles with the following conditions: denaturation (94°C for 30 seconds), annealing (60°C for 30 seconds), and extension (72°C for 30 seconds). A final extension at 72°C for 5 minutes was performed. All products were electrophoresed on a 2% agarose gel, which was stained using a SYBR Safe DNA solution (Invitrogen, Carlsbad, CA, USA).

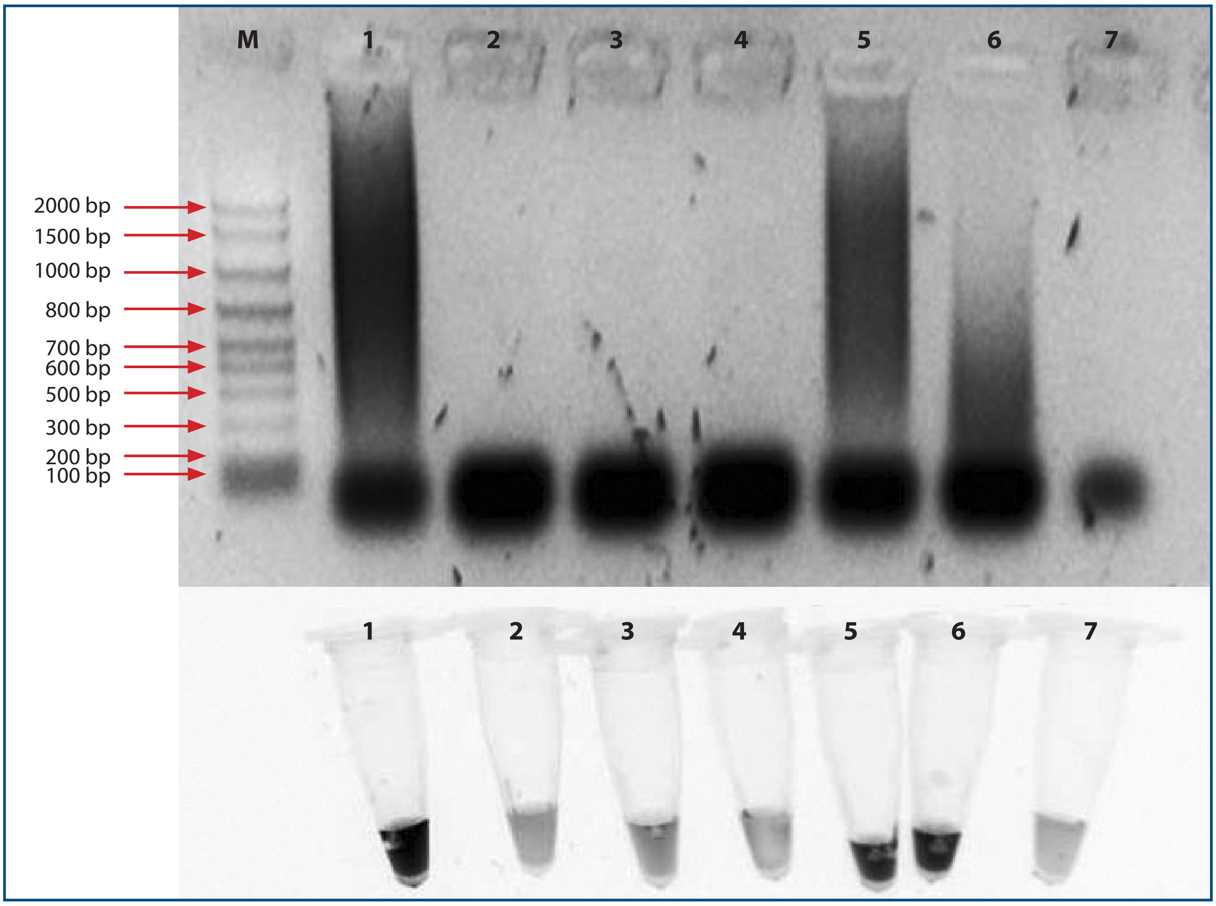

Figure 1. *Presence of representative DNA products: LAMP amplicons detected on a 2% agarose gel (upper panel) and after addition of 1 µl of 1:10-diluted propidium iodide to the mixture (lower panel).* Lane M, DL 2000 bp ladder marker; lane/tube 1: positive goat placenta; lane/tube 2: negative sheep blood; lane/tube 3: *Rickettsia helvetica*; lane/tube 4: *Chlamydophila abortus*; lane/tube 5: positive buffalo cotyledons; lane/tube 6: *C. burnetii* positive control (Nine Mile strain); lane/tube 7: negative sample (nuclease free water). Positive samples (1, 5, 6) showed a characteristic ladder pattern (upper panel) or a brilliant fluorescence (lower panel), while negative samples showed no fluorescence.

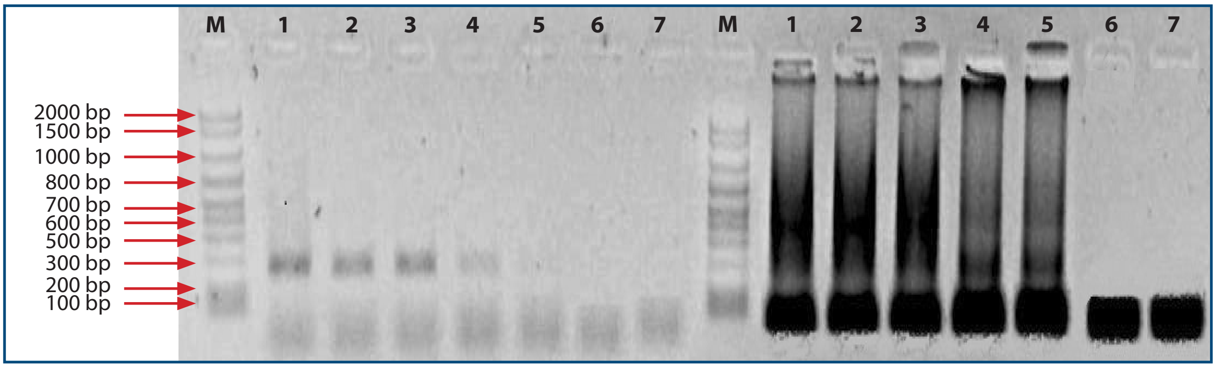

Figure 2. *Comparison of the detection limit of our PCR targeting* com1 *gene (on left side) and LAMP (on right side): Lanes M, DL 2000 bp ladder marker, lanes 1: 100ng, lanes 2: 10ng, lanes 3: 1ng, lanes 4: 100pg, lanes 5: 10pg, lanes 6: 1pg and lanes 7: 100fg indicate DNA concentration/ml.*

Results

The LAMP assay correctly identified all the clinical specimens containing *C. burnetii* (Table II) and its products showed a characteristic ladder pattern on a 2% agarose gel (Figure 1). LAMP assays of genomic DNA from the microorganisms and from the negative controls were negative (Table II); UV illumination of the reaction tubes containing the positive controls produced brilliant fluorescence, whereas the tubes containing the negative controls did not show any fluorescent signal (Figure 1). Both our LAMP assay and our PCR assay detected *C. burnetii* DNA to the same limit of detection equal

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to 10 pg DNA/µL (Figure 2), although the LAMP gel pattern was stronger than the PCR pattern.

Discussion

The epidemiological situation of Q fever differs considerably across Europe, and to date it is poorly known in Italy. The disease often spreads unsuspected areas, as it was the case in the Netherlands in the 2007 (Roest *et al.* 2011). Domestic ruminants are considered the main reservoir of human Q fever, thus diagnosis is crucial in the animals to hopefully prevent human outbreaks. Q fever is usually diagnosed by serological testing, which is not suitable for early diagnosis, because of the delay in appearance of diagnostic antibodies (Wegdam-Blans *et al.* 2012). In this study, we developed a molecular assay able to rapidly detect *C. burnetii* DNA directly from clinical samples of abortive material without attempting any microbiological isolation, which requires a well-equipped biosafety level 3 laboratory. In contrast, LAMP can be performed in a conventional diagnostic laboratory and even in the field using portable instruments. This test does not require any specific or expensive equipment. It only requires a heating block, which is readily available in most research and diagnostic laboratories. Furthermore, products are easily visualized using UV light and even by naked eye with either turbidity or colour changes. These features enable testing in a large variety of laboratories and make LAMP a promising platform for the molecular detection of important zoonotic infections in developing countries.

Based on levels of 10^9 *C. burnetii*/g in abortion products reported by Maurin and Raoult (1999), the limit of detection of 10 pg revealed by *com1* LAMP is appropriate for such testing. The total time required for the LAMP assay, which included amplification and detection, was about 60 minutes, whereas conventional PCR required 3 hours.

Although relatively few clinical samples were tested, these preliminary data suggest that the *com1* LAMP assay was both sensitive and specific. We did not detect any cross-amplifications in the negative tissue controls. Similarly, cross-amplifications were not detected in the large bacteria collection analysed for the most prevalent abortion agents such us *Brucella melitensis, B. abortus, Chlamydophila abortus* and *Salmonella*. The differential diagnosis of contagious abortions is essential in Italy, where for example, brucellosis still remains prevalent and responsible of superimposable clinical outcomes (Garofolo *et al.* 2013).

The insertion element IS1111 is the most commonly used target sequence for the molecular detection of *C. burnetii* likely due to its high copy number enhancing detection. However, other different targets have been used such as *icd*, *com1*, and *sod* (de Bruin *et al.* 2011, Klee *et al.* 2006). The LAMP assay was developed with *com1*, because this gene is considered highly conserved in *C. burnetii*; and being it present in a single copy, it permits a more accurate quantification of the pathogen load in the samples. The use of *com1* gene instead of the IS1111 should be considered as a reliable alternative and further examinations of performance parameters such as efficiency, reproducibility, and repeatability warrant additional study.

In conclusion, *com1* LAMP assay is another rapid, specific, economic, and simple method for the identification of *C. burnetii* DNA. It has the sensitivity comparable to a standard PCR assay. Practicing veterinarians may use this technology in the field to investigate any suspicious abortion to better address the management of coxiellosis.

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