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Original Contribution

LOOP-MEDIATED ISOTHERMAL AMPLIFICATION FOR DETECTION OF *C. BURNETII* IN BULGARIA

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ABSTRACT

Q fever, which is caused by *Coxiella burnetii*, a small, pleomorphic intracellular bacterium, is the most widespread zoonosis in the world. The chronic form of the disease can lead to disability and death. Rapid diagnosis of Q fever is needed in order that effective treatment can be initiated. The conventional retrospective diagnosis of Q fever, based on serology, is useless for the treatment of afflicted patients. Thus, molecular methods have been created to close the diagnostic gap between the onset of the disease and the presence of specific antibodies in serum. A polymerase chain reaction is a suitable and reliable method with high sensitivity and specificity, but it requires expensive equipment and post-amplification protocol. Loop-mediated isothermal amplification (LAMP) is an isothermal technique, conducted at constant temperature that can amplify a negligible amount of DNA to more than 10^9 copies within one hour, using special primers and polymerase. We have tested the sensitivity and specificity of LAMP in the detection of *C. burnetii.* The mean positive rate of LAMP and polymerase chain reaction in patients was 100% and 74%, respectively. LAMP reacted negatively with non-*C. burnetii* pathogens and non-infected blood samples. We conclude that LAMP is a sensitive and specific technique for the detection of *C. burnetii* and has advantages over serological methods and PCR that make it attractive for diagnosing Q fever in countries around the world.

Key words: Loop-mediated isothermal amplification; polymerase chain reaction; Coxiella burnetii; Q fever; Bulgaria

INTRODUCTION

Q fever, the most widespread zoonosis in the world, is caused by *Coxiella burnetii*, a small, pleomorphic intracellular bacterium. The usual source of human infection is farm animals (cattle, goats, sheep, and pets), cats and dogs. *C. burnetii* is shed in feces, urine, and milk and especially in birth products. The reservoir of *C. burnetii* is mammals, birds, and ticks. In Bulgaria, *C. burnetii* has an endemic spread.

Expression of Q fever in humans may be either acute or chronic. Acute Q fever is a flu-like illness, which usually is self-limited or easily treated with antibiotics when the diagnosis is correctly made. However, chronic Q fever is a severe disease that requires prolonged antibiotic therapy in order to prevent complications such as endocarditis and granulomatous hepatitis.

Because of its long persistence in the environment, low infectious dose, and the possibility of aerosol transmission, *C. burnetii* has been classified as a category B bioterrorist agent by the United States Center for Disease Control and Prevention (1-3). Rapid identification of *C. burnetii* in clinical specimens is very important for the control of Q fever because prompt antibiotic therapy may lead to better patient outcomes.

The diagnosis of Q fever both in humans and animals is made mainly on the basis of serological testing (indirect immunofluorescence, complement fixation, and enzyme-linked immunosorbent assay) (4). A drawback of these techniques is that

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establishment of the diagnosis is slow because specific antibodies appear only several weeks after infection, although they can be detected for months thereafter. For this reason, the serological tests offer only a retrospective diagnosis and are of no value in the decision-making of whether afflicted patients should be treated; only when the diagnosis is made early can effective treatment be instituted, a necessary step if chronic disease with the attendant disability and, possibly, death is to be averted.

For early diagnosis of Q fever, polymerase chain reaction (PCR) is a suitable and reliable method with high specificity and sensitivity (5, 6). However, PCR has drawbacks: the need for sophisticated instruments in order to maintain various temperatures for various times; the need for post-amplification protocols, such as for electrophoresis; the requirement of a three- to four-hour period to obtain results; and the need for extraction of DNA from samples before the PCR can be carried out (7). Also, real-time PCR tests can be conducted only in well-equipped laboratories because of the need for expensive instruments and skill required for interpretation of the results.

Aim

Among the isothermal amplification techniques, the unique loop-mediated isothermal amplification (LAMP) technique has attracted attention (8-15). Our aim in this study was to determine the reliability of the LAMP assay, compared with conventional PCR, with both methods targeting the *C. burnetii* IS *1111a* insertion element, for rapid, sensitive, and specific diagnosis of *C. burnetii* in Bulgarian patients.

MATERIALS AND METHODS

For studies on human subjects, blood samples were collected from fourteen male and nine female Bulgarian patients with the clinical diagnosis of acute Q fever during 2007-2016. DNA was extracted from human blood samples by use of QIAmp blood kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

We used "in-house" conventional PCR. One pair of specific primers were designed, based on inserting sequence *IS 1111a* of *C. burnetii* genome. The forward primer was IS1trg-f: AGAATTTCTATTTTCAAAAAAAGGAGA AG; the reverse primer was IS1trg-r: CGGTTCAACAATTCGGTATACAAACAA. Product length was 605 bp (Qiagen, Wesburg, Germany).

The general PCR with primer pairs IS1/IS2 was performed in multiple 25 μl reaction volume sets containing 0.2 μM of each of the primers, together with 0.4 mM of deoxynucleoside triphosphate, and 1 U/ μl of recombinant *Taq* DNA polymerase. We cycled the PCR reaction 35 times with a denaturation step of 94^0C for a period of 40 seconds followed by annealing at 50^0C for 30 seconds and extension at 72^0C for 40 seconds. Finally the products of the reaction were specially electrophoresed on a 1% (TBE – Tris, Boric acid and EDTA) agarose gel and stained with ethidium bromide (1 μg/ml) as described (16). The reaction was performed in a Mastercycler (Eppendorf, Germany).

LAMP Primers Design

A special set of universal primers against the repetitive sequence *IS 1111a* of the *htpAB* gene of *C. burnetii* RSA 493 (AE016828) was designed and prepared by use of Primer Explorer V4 software (Eiken Chemical Co., Ltd., Tokyo, Japan, *http://pimerexplorer.jp*) based on conserved sequences that were determined by aligning 5 *htpAB* gene GenBank entries. Primers were synthesized by the biotechnology company Sangon Biotech (Shanghai, China). On the next figure, it is presented the primer sequences and their positions relative to the *htpAB* gene:

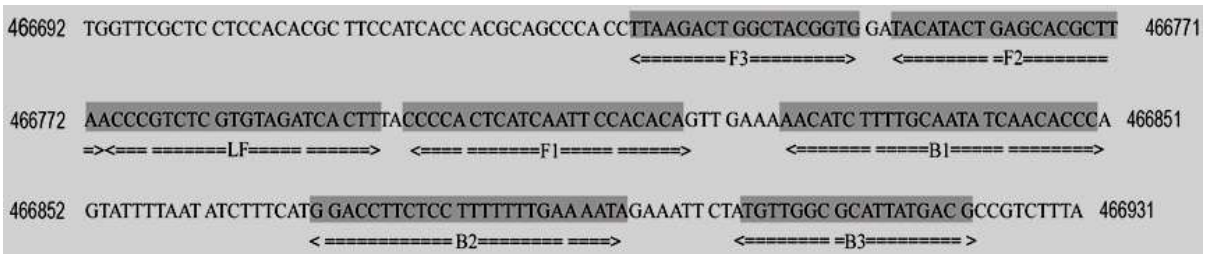

Figure 1. Names and binding sites about the primers for LAMP related to Q fever

The LAMP Reaction

All of the LAMP reactions were realized with the product *Loopamp amplification Kit* (developed by Eiken Chemical Co., Ltd., Tokyo, Japan) in a 25 µl reaction volume containing 1.6 µM of each of the FIP and BIP primers, 0.8 µM of the LF and LB primers, 0.2 µM of the F3 and B3 primers, 20 mM Tris-HCL (pH 8.8), 10 mM KCL, 8 mM $MgSO_4$, 10 mM $(NH_4)_2$ SO_4, 0.1% Tween-20, 0.8 M betaine, and 1.4 mM of each deoxynucleoside triphosphate (dNTP) and 1µl *Bst* DNA polymerase (320U/ml). The reaction was incubated in a water bath at 63^0C for a period of 60 min and then at 80^0C for 5 min of time to terminate the reaction. After the performed amplification, the LAMP products were consequently visualized with some *hydroxyl-naphthol blue staining*; the appearance of violet color was considered for a negative result and the sky blue colorification as a positive result.

Evaluation of the Sensitivity of the LAMP Assay

To compare and assess the sensitivity of the *htpAB* LAMP assay and general PCR, the reference plasmid, which was serially diluted (at concentrations of 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 copies/µl), containing the target DNA, was used to first define the limit of detection, as described before (16).

Evaluation of the Specificity of the LAMP Assay

To determine the specificity of the LAMP assay for *C. burnetii,* we investigated DNA extracted from the bacterial pathogens *Rickettsia rickettsii, Rickettsia conorii, Rickettsia sibirica, Escherichia coli, Legionella pneumophila,* and *Yersinia pestis EV Mollerat*, as well as from non-infected human blood samples.

RESULTS

Sensitivity of LAMP

We determined that the limits of detection provided by LAMP and PCR for the *htpAB* gene were in the borders between 1 and 100 copies per reaction, respectively. Thus, the LAMP assay is 100-fold more sensitive than PCR at detecting *C. burnetii*.

Examination of blood samples from patients with acute Q fever

LAMP and PCR were compared under identical conditions, using extracted DNA from blood samples of patients with acute Q fever, documented with positive *C. burnetii*–II phase IgM antibodies in ELISA, 23/23 (100%) were positive in LAMP and 17/23 (74%) in PCR; all 10 healthy

Table 1. *Results, obtained from investigation of blood samples of patients with acute Q fever and healthy persons with LAMP and PCR persons were negative in both LAMP and PCR(Table 1).*

Blood samples	Number	ELISA IgM		LAMP			PCR		
		(+)	(-)	(+)	(-)	%(+)	(+)	(-)	%(+)
Acute Q-fever	23	23	0	23	0	100	17	6	74
Healthy persons	10	0	10	0	10	0	0	10	0

The positive rate of LAMP and PCR for *C. burnetii* was 100% and 74%, respectively, a statistically significant difference ($p<0.01$). Similar limit of detection (LOD) and sensitivity have been reported by others (13).

Specificity** of **LAMP

The examined by the investigators six common pathogens did not reveal positive for *C. burnetii* with LAMP. This result provides evidence of specificity of the *htp*AB LAMP assay for Q fever.

CONCLUSION

This work was designed to develop a sensitive and specific test for *C. burnetii*, the organism causing Q fever. We began by choosing a conventional PCR with primers pair targeting the transposase gene. The transposase gene in insertion element IS *1111a* is a preferred target for PCR assay due to its presence in multiple copies within the genome of *C. burnetii*, thereby enhancing the sensitivity of detection(1). We then used *htp*AB LAMP with primers targeting the same gene and found that it is 100 times more sensitive than is PCR.

The difference in sensitivity of the two tests may have various explanations: 1) LAMP uses very specific primers, based on eight target regions present on the gene. 2) LAMP is carried out with DNA polymerase, which has

the unique property of strand displacement as well as the usual polymerase activity; thus LAMP can amplify a negligible amount of DNA to more than 10^9 copies within one hour. 3) The greater concentration of amplification products allows easier visualization of the result.

Following the experience of the Chinese group of Pan Lei et al, 2013, we applied LAMP for the diagnosis of *C. burnetii* infection for the first time in Bulgaria. Using a referent recombinant plasmid, we established that LAMP is 100-times more sensitive than PCR, with the limit of detection 1 and 100 copies per reaction, respectively. We tested the method on the model in patients with acute Q fever, finding that LAMP is more sensitive than PCR for detection of *C. burnetii* in blood samples; the mean positive rate of LAMP and PCR was 100% and 84.6%, respectively. These results are similar to those of Pan Lei et al, 2013, who detected *C. burnetii* for the first time with LAMP. We also documented the specificity of the LAMP assay for *C. burnetii* by showing that it did not react with non-*Coxiella* pathogens and non-infected blood.

An additional advantage of the LAMP assay is that it is conducted at isothermal temperatures and does not require sophisticated equipment and skilled personnel. Thus, it is well suited for use in countries with limited resources.

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