EFFECTIVE LUX (Light Upon eXtension) PRIMER SYSTEM FOR EARLY AND RAPID DETECTION OF Coxiella burnetii

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ABSTRACT
Coxiella burnetii is the cause for Q-fever that is spread all over the world and affects both, animals and humans. Q fever is a disease of great significance which may take a chronic or even lethal course. Coxiella burnetii is a potential biological agent that is possible to be used as a biological weapon. Prompt and specific diagnostic tools as well as elaborating new detection methods are needed for an exact diagnosis and a correct etiological treatment. One of the most contemporary molecular – biological methods for a prompt and specific detection at genetic level is the real-time Polymerase Chain Reaction (PCR). LUX primers are used instead of conventional ones and a new technology for olygonucleotides marking is applied. We have constructed LUX Primer System with the support of D-LUX TM Designer and have successfully tested its specificity, sensitivity and effectiveness in sequence detecting of Coxiella burnetii genome which is strongly peculiar to that kind of microorganism.

Keywords: real-time PCR (Polymerase Chain Reaction), LUX (Light Upon eXtension) primer system, Coxiella burnetii

Introduction
Coxiella burnetii is an obligatory intracellular gram-negative bacterium which develops in cell culture, hen embryo and guinea pigs, but cannot be grown in axenic medium (15, 11, 18, 19, 23). It belongs to a gamma–partition of Proteobacteria, Legionellales order, Coxiellaceae family, and Coxiella genus (3). The size of the genome varies between different Coxiella burnetii strains, from 1,5 to 2,4 Mb. It is considered to have a circular BI-chained DNA and there are 4-plasmide types depending on the different kind of strains: QpH1, QpRS, QpDG, QpDV (18). Coxiella manifests a high level of steadiness in external environment. A lot of mammal, bird and tick species can be a reservoir for nurturing of that kind of microorganism (1, 18). The infection caused by Coxiella burnetii most often takes a latent course in animals constantly eliminating the bacterium in the surrounding area.

Coxiella burnetii can enter the human organism through the aerosol (the most important way) (16, 22); through the alimentary tract by absorption of an overdose of the microorganism with the food and water (4, 9, 14); the transmission vector way (tick) (8); congenital infection via placenta (21); blood transfusion (2); sexual contact (17); skin and mucosa are of least significance (via contact) (2, 10, 16, 20).

Q fever is an infection that shows a varied clinical picture and the lack of specific features hampers its diagnosis, prevention and correct etiological treatment. Coxiella burnetii has two manifestations – acute (atypical pneumonia and hepatitis) or chronic (endocarditis). Nearly 60% of the cases take an asymptomatic course (1, 17). During pregnancy the disease can lead to abortion or prematurely born fetus (21).

For the Coxiella burnetii detection a complex of methods for lab diagnosis, including: isolation (most often of cell cultures, hen embryos) (24), serology (the immune – fluorescent method, as a golden standard, ELISA and etc.) (18), molecular-biological methods (conventional PCR, real-time PCR) are used (6, 7, 12, 13). Coxiella burnetii isolation must be performed only in the set of high bio-security laboratories, level 3. The diagnosis of Coxiella burnetii via direct and indirect immune-fluorescent method is proved to be referent and has been used most often. Our attention is drawn at the perspective molecular - biological methods that are currently going through a rapid development and their significance in detection of that microorganism is increasing. These complement the complex of methods by their features: promptness, sensitivity, and specificity. One of these molecular – biological methods for a rapid and specific detection at genetic level is the real-time PCR. LUX technology represents a new type for primers design and labeling. These fluorogen primers are produced as a pair of 2 primers. The first primer is marked by one fluorofore, i.e. FAM (6-carboxyfluorescin), the second one is not marked. Due to the specific conformation of the marked primer as “hairpin”, an interior fading of the fluorofore occurs. The connection of primer to the specific genome section prolongs the chain length and leads to enhancement of fluorofore fluorescence as a result.

From a military medical point of view it is important to be mentioned that Coxiella burnetii is a potential biological agent possible to be used as a biological weapon. It belongs to B category (Center for Disease Control and Prevention) as a bio-agent with less dissemination, causing infections of medium severity with less mortality, but requiring an enforced diagnosis capacity from CDCP and undivided attention.

Prompt and specific diagnostic tools as well as elaborating new detection methods are needed for an exact diagnosis and
a correct etiological treatment. If the disease is not treated correctly, it may take a severe, chronic and even lethal course.

**Materials and Methods**

We used the genome of *Coxiella burnetii* RSA 493 with length 1995281 nt, Accession number 16828 in the gene bank, refereq: NC_002971, published in: http://www.ncbi.nlm.nih.gov/Genbank. It consists of 2143 genes, coding 2016 proteins for developing Light Upon eXtension (LUX) primer system. We have carefully selected the gene (sodB), coding strictly specific protein superoxide dismutase (fe). On the base of that gene we have chosen and applied to 10 equal DNA samples positive for *Coxiella burnetii*. Optimization of the work protocol on the base of obtained results was made.

Determination of LUX primers specificity was done by real-time PCR with control genomes of both phylogenetically close and distant microorganisms - *Escherichia coli* (ATCC® 25922), *Legionella pneumophila* (ATCC® 33152), *Chlamydia psittaci* (ATCC VR 125), *Rickettsia rickettsii* (ATCC VR 891), *Rickettsia prowazekii* (ATCC VR 142) and *Rickettsia sibirica* (ATCC VR-1526T).

**LUX Primers Sensitivity.** Sensitivity estimation of LUX Primers was performed by real-time PCR with different DNA concentrations extracted from purified *Coxiella burnetii* corpuscular antigen, strain Heinzerling.

All LUX real-time PCR were performed at least three times for reliability.

**Results and Discussion**

The used D-LUX™ Designer software for generating LUX Primer Pairs has 20 different possible combinations. As a result of detailed analysis of different indexes: specific conformation, guanin-cytosin content 50 - 60% and similar hybridization temperature a particular pair named CBL1 (FAM)/CBL2 was chosen. The work protocol for these primers was optimized using positive *Coxiella burnetii* corpuscular antigen, strain Heinzerling.

The work protocol includes initial denaturation at 95°C per 10 min; 50 cycles including consecutively 95°C per 10 sec. and 60°C per 1 min, with estimation of the fluorescence at each cycle. The further steps include incubation at 95°C per 1 sec. curve of melting at which amplification products are exposed on temperature amplitudes of 60 - 95°C, estimating fluorescence every 0.2°C. The protocol is completed with incubation at 25°C within 30 min, following of decreasing to 4°C.

**Table 1**

<table>
<thead>
<tr>
<th>Name</th>
<th>The gene encoding</th>
<th>Sequence (5’ to 3’)</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBL1(FAM)</td>
<td>superoxide dismutase (fe)</td>
<td>CGC ACG CTT CCA GTT CCA GTT TGT TTT G[FAM]G</td>
<td>24</td>
</tr>
<tr>
<td>CBL2</td>
<td>superoxide dismutase (fe)</td>
<td>ATC AGA GCG GAC CGT CAA GC</td>
<td>20</td>
</tr>
</tbody>
</table>
LUX Primer system CBL1(FAM)/CBL2 shows high sensitivity and specificity during the trials.

**LUX Primer Specificity:** This was tested by real-time PCR with genome control samples of both: phylogenetically close and distant microorganisms - Escherichia coli (ATCC® 25922), Legionella pneumophila (ATCC® 33152), Chlamydia psittaci (ATCC VR 125), Rickettsia rickettsii (ATCC VR 891), Rickettsia prowaceki (ATCC VR 142) and Rickettsia sibirica (ATCC VR-1526T). As a result of the study (Fig. 1a) no specific amplification except for the positive control sample, containing Coxella burnetii DNA (line 1) was shown, which proves the unique value of The LUX Primer System.

**LUX Primers Sensitivity.** The sensitivity estimation of LUX primers was accomplished through real-time PCR with different DNA concentrations (Fig. 1b) extracted from purified Coxella burnetii corpuscular antigen, strain Heinzerling. DNA concentration was measured by Bio Photometer (Eppendorf), and an extraction of 58.7 ng/μl was reported. Concentrations of DNA were used as follows: 5.87 ng/μl – diluted 1:10 (line 1); 0.587 ng/μl - diluted 1:100 (line 2); 0.0587 ng/μl - diluted 1:1000 (line 3); 0.00587 ng/μl - diluted 1:10,000 (line 4) and a zero check (line 5). The high LUX Primers sensitivity is evident as it detects DNA concentration in 0.587 ng/μl - diluted 1:100.

To sum up, the constructed LUX Primer system is specific and sensitive and can be successfully used together with the complex of diagnostic methods for rapid detection of Coxella burnetii.

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**REFERENCES**