

Our experience using real-time PCR for the detection of the gene that encodes the superficial lipoprotein LipL32 of the pathogenic leptospires to confirm the acute form of human leptospirosis

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Aims. To examine biological materials (blood, urine, cerebrospinal fluid) of patients with suspected leptospirosis using real-time PCR for detecting the gene that codes the superficial LipL32 lipoprotein, and to evaluate the contribution of the real-time PCR method for the laboratory diagnosis of the acute form of leptospirosis.

Material and Methods. During the monitored period of April 2010 – December 2011, a total of 340 biological materials samples were examined (177x blood plasma, 88x urine, 68x, cerebrospinal fluid, 6x bronchoalveolar lavage and 1x sputum) from 216 patients with suspected leptospirosis using real-time PCR LipL32 gene detection.

Results. From the mentioned 216 patients suspected of leptospirosis, 8 patients were evaluated as being PCR LipL32 positive, from which 14 positive biological materials originated (9 x urine, 4x blood and 1x liquor).

Conclusion. As demonstrated in the study, the real-time PCR method for detecting the gene for the superficial lipoprotein LipL32 is an appropriate, quick and reliable method for the diagnosis of the acute form of leptospirosis.

Key words: leptospirosis, real-time PCR, LipL32, MAT

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INTRODUCTION

Leptospirosis is a typical zoonosis with a worldwide distribution; a higher prevalence of disease is traditionally described in geographical areas with moister and warmer climates – in the tropics and subtropics, where environmental and socioeconomic conditions are suitable for the transmission of the disease¹⁻³. The infective agents are aerobic spiral bacteria of the *Leptospira* genus which, according to serological classification, includes more than 300 serotypes of pathogenic leptospires. On the basis of genetic analysis, leptospires are divided into 20 genomospecies, pathogenic leptospires fall into 13 genetic types^{4,5}.

The disease is usually transmitted by contact with water or moist substrates that have been contaminated by the urine of reservoir animals, especially of rodents, or after coming in contact with the animal itself. Veterinarians, workers in sewers and drainages, workers in slaughterhouses and in agriculture have a higher risk of infection from exposure to the pathogenic leptospires. Professional infection represents 30-50% of cases of human leptospirosis⁶⁻¹⁰. The occurrence of leptospirosis is fundamentally influenced by two phenomena – floods and rodents overbreeding. After floods, local epidemics of leptospirosis are often described⁷.

The incubation period ranges from 1-4 weeks. The disease can proceed with slight influenza symptoms up to states with hepatorenal failure, cardiac and respiratory insufficiency, haemorrhagic diathesis or aseptic meningitis^{1,6,7,11}.

Laboratory diagnosis depends on the typical two-phase course of the disease. In the first week of clinical symptoms, when antibodies have not yet been produced, it is recommended to examine the material obtained from patients by molecular biological methods.

In the second week of the disease, special serological methods are used – the Microscopic Agglutination Test (MAT). In many publications, diagnostic ELISA kits for detecting IgM antibodies to confirm the acute form of leptospirosis are used.

In our study, we focused on detecting the acute form of leptospirosis – by detection of the gene that encodes the superficial LipL32 lipoprotein of molecular weight of 32 kDa and made up of 251 amino acids¹². It is present only in pathogenic leptospires and its structure is highly conservative (conformity in the primary sequence aminoacids of up to 98%). It is the main protein component of the outer membrane of leptospires (more than 75% of the whole weight of proteins). In vitro, it is produced during cultivation, but also during the acute or chronic

phases of the disease. LipL32 is highly immunogenic, it has been demonstrated that more than 95% of patients with leptospirosis produce antibodies against this antigenic determinant¹³. It has also been found that this lipoprotein acts as an adhesin which bonds to proteins of host cells – collagen I, collagen V, laminin, collagen IV and plasma fibronectin^{14,15}. It potentiates haemolysis mediated by sphingomyelinase SphH and, for this reason; it is classified as hemolysis-associated protein Hap-1. It induces an anti-inflammatory response in renal cells and promyelocytic cell line CD14 (ref.^{16,17}).

In 2010, the real-time PCR method for detecting the gene that encodes the superficial lipoprotein LipL32 was introduced at our collaborating centres at the Department of Clinical Microbiology and the Department of Clinical Biochemistry and Diagnostics Faculty of Medicine in Hradec Kralove and University Hospital in Hradec Kralove.

Aims of study

1) To examine biological materials (blood, cerebrospinal fluid, urine, etc.) from patients suspected of leptospirosis using real-time PCR in order to detect the gene that encodes the superficial lipoprotein LipL32.

2) To investigate the blood samples using the MAT method to confirm the results of PCR examination.

3) To evaluate the contribution of real-time PC in the laboratory diagnosis of acute leptospirosis.

MATERIALS AND METHODS

Biological materials

In the monitored period of April 2010 – December 2011, 340 biological materials (177x blood plasma, 88x urine, 68x cerebrospinal fluid, 6x bronchoalveolar lavage and 1x sputum) from 216 patients with suspected leptospirosis were examined at our institution, using real-time PCR to detect the gene for the superficial lipoprotein LipL32. This analysed set of samples was obtained from 140 men and 76 women. The average age of the men was 44, 94 ± 20, 16 (age range 1 - 88), the average age of women was 44, 83 ± 19, 68 (age range 7 - 87), the average age of men + women of 44, 90 ± 19, 99 (age range 1 - 88).

The informed consents of the patients are saved in the Department of Clinical Microbiology in Hradec Kralove.

Concentration of biological materials

Biological materials were first concentrated by the methods used and validated in our department. These increase the limit of detection to more than 1 - 5 copies of genome in 1 mL of the liquid biological material.

Urine: 10-15 mL of urine was centrifuged at 2 500 rpm, the supernatant was sucked up and sediment (about 1.4 mL) was transferred in a 1.5 mL test-tube and centrifuged at 13 000 rpm. Supernatant was removed and the pellet was elaborated according to the kit instructions.

Blood plasma, liquor: 0.5 to 1.5 mL of plasma or liquor were centrifuged for 15 min at 13 000 rpm, using a refrigerated centrifuge. 200 µL of distilled water was added to

the supernatant, the mixture was mixed and centrifuged for 8 min at 13 000 rpm (at 4 °C).

The supernatant was removed and the pellet was processed.

The isolation of DNA from biological materials of patients (non-coagulated blood, urine, cerebrospinal fluid) was performed by the kit QIAamp DNA Mini Kit (QIAGEN, Roche, Czech Republic).

The real-time PCR method

For the detection of DNA pathogenic leptospires using the in-house real-time PCR (SybrGreen I) on the Lightcycler 1.5 amplification primers LipL32-270F (25 bp) and LipL32-692R (26 bp) from the area of the gene for LipL32 (amplification product of the size of 400 bp) were applied¹⁸. As a control for inhibition of DNA polymerase, amplification of the housekeeping gene for β actin during usage of primers IC838-2 IC (32 bp) and IC838-1 IC (32 bp) was done and an amplification product of 838 bp was obtained.

The on-line record of fluorescence of the product at a properly selected amplification temperature profile was realized at the end of every cycle at the wave length λ = 530 nm, the identification of the presence of DNA pathogenic leptospires was performed by the using of the analysis of the temperature of melting.

Serological confirmation by the MAT method

The detection of antibodies against pathogenic leptospires was carried out in 145 patients (177 blood samples) using the MAT method with 11 laboratory strains used in the Czech Republic: *L. Icterohaemorrhagiae* Fryšava, *L. Copenhageni* Lebe, *L. grippityphosa* P125, *L. grippityphosa* Ž6, *L. sejroe* M64, *L. isticra* J20, *L. Bratislava* Jež Bratislava, *L. Pomona* Šimon, *L. polonica* Poland, *L. canicola* S392 and *L. sorex-jalna* Sorex jalna. Titration was performed from dilution 1:50 with using binary dilution.

A positive result of the test, the titre of 1:400 and more was considered, as a borderline, the titre of 1:200 (ref.¹⁹).

RESULTS

Of 216 patients examined with the real-time PCR method for detecting the gene LipL32, 8 (3.70%) persons were evaluated as being leptospira positive; of these 14 (4.12%) positive biological materials were gained (9x urine, 4x blood and 1x liquor). All positive biological materials were sampled in term when no sufficient titre of specific antibodies was obtained and the results of microagglutination test were negative (in the first week of the disease when clinical symptoms in patients were present and anamnestic data led practitioners to suspect leptospirosis).

According to the requirements of the clinicians, blood sera of 145 patients (177 samples) were examined using the MAT method. In 8 PCR positive patients (Table 1), a negative MAT result was noted 3x, a border result 3x and 1x positive. In one case, the examination was not carried out. Of the remaining 137 patients examined serologically,

Table 1. Results of examination with using PCR and MAT methods of positive patients.

Results of examination	1 st examination	2 nd examination	3 rd examination	4 th examination	5 th examination
Patient no. 1	PCR 21.6.2010 blood - pos., urine - pos.	-	-	-	-
Patient no. 2	MAT 29.6.2010 <i>L. sorex-jalna</i> 1:50	PCR 29.6.2010 blood - pos., urine - pos.	MAT 7.7.2010 <i>L. grippotyphosa</i> Ž6 1:200	-	-
Patient no. 3	MAT 28.7.2010 <i>L. bratislava</i> Jež Bratislava 1:200	PCR 28.7.2010 blood - pos., urine - pos.	PCR 11.8.2010 urine - neg.	-	-
Patient no. 4	PCR 4.8.2010 blood - pos., urine - pos., liquor - neg.	MAT 5.8.2010 neg.	-	-	-
Patient no. 5	MAT 23.8. 2010 <i>L. icterohaemorrhagiae</i> Fryšava 1:50	PCR 23.8.2010 blood - neg., urine - pos. sputum - neg.	-	-	-
Patient no. 6	PCR 25.8.2010 blood - pos., urine - border	MAT 26.8.2010 <i>L. copenhageni</i> Lebe 1:50 <i>L. sorex-jalna</i> 1:50	PCR 1.9.2010 urine - neg.	MAT 3.9. 2010 <i>L. grippotyphosa</i> P Ž6 1:400 <i>L. grippotyphosa</i> P125 1:200	MAT 1.10. 2010 <i>L. grippotyphosa</i> P Ž6 1:800 <i>L. grippotyphosa</i> P125 1:400
Patient no. 7	PCR 25.11.2010 blood - pos., urine - pos.	MAT 14.1.2011 <i>L. grippotyphosa</i> P125 1:200	-	-	-
Patient no. 8	PCR 12.9. 2011 blood - neg, urine - pos.	MAT 12.9.2011 <i>L. grippotyphosa</i> P Ž6 1:50 <i>L. grippotyphosa</i> P125 1:50 <i>L. sejroe</i> M64 1:50	-	-	-
Patient no. 9	PCR 9.9.2010 blood - neg, urine - neg	MAT 29.9.2010 <i>L. copenhageni</i> Lebe 1:400 <i>L. grippotyphosa</i> Z6 1:100 <i>L. sejroe</i> M 64 1:100	-	-	-
Patient no. 10	PCR 23.9.2011 blood - neg, urine - neg	MAT 21.10.2011 <i>L.sejroe</i> M64 1:800 <i>L.istrica</i> J20 1:1600 <i>L.polonica</i> Poland 1:6400	-	-	-

Legend: PCR - polymerase chain reaction, MAT - microagglutination test, pos. - positive, neg. - negative

antibodies against *L. Polonica* Poland in titre 1: 6400 were demonstrated in one patient and a slightly positive titre (1:400) against *L. Icterohaemorrhagiae* Fryšava, in one patient too. For both patients, the PCR results were negative.

DISCUSSION

As we consider the PCR method crucial in the early diagnosis of acute leptospirosis, we analysed the results of the examination of all patients with positive PCR as well as both MAT positive/PCR negative persons.

Biological materials from patients No. 2, 3, 4, 5, 6 and 8 were taken for PCR and MAT examinations on the same day or at an interval of 24 h (Table 1). This fact supports the importance of PCR for the early laboratory diagnosis of leptospirosis as the detection of antibodies in patients with positive PCR results was, using the MAT method, at the same time negative in 3 patients and borderline at 3 patients. Serological laboratory methods only would thus not reveal at the given time, acute leptospirosis in any of the six patients.

Samples from patient No. 7, in which PCR positivity was demonstrated both in blood and urine, were sent by the clinical institution for MAT examination as late as 2 months later. The MAT result was borderline, showing a titre of 1:200.

To assess the contribution of PCR in the diagnosis of acute leptospirosis, the biological materials of patients with negative results for detection of DNA and serological positivity should also be considered.

Blood and urine from patient No. 9 were evaluated on 9th September as LipL32 negative. After less than 3 weeks, a positive MAT result was noted, that is, 1:400. This was confirmed by the attending physician: on 7th September, that is two days before sampling of biological materials for the detection of DNA, antibiotic treatment was started. This may explain the negative result of PCR.

Biological materials from patient No. 10 (blood and urine) were PCR LipL32 negative on 23rd September. After less than a month, a positive MAT result was noted in three laboratory strains of pathogenic leptospires (the highest titre 1:6400). According to the clinic, the patient was not on antibiotics, at the time of blood and urine collection for PCR examination. For this reason, we assumed that there were already antibodies and hence no leptospires would be found in the blood. Colonization of the renal tubules usually occurs after 7 days of the initial leptospiremia and, hence positivity in the urine can be expected. The negative result of PCR urine therefore led us, to another investigation at the clinical institution where we confirmed that the urine taken on 23rd September was processed as late as 26th September.

In our previous study we determined the diagnostic sensitivity and specificity of the diagnostic kit SERION ELISA classic IgM/IgG leptospira (Serion, Germany). In both ELISA kits detecting IgM and IgG antibodies against pathogenic leptospires a diagnostic sensitivity of 100%

and diagnostic specificity of 88.6% and 54.3% respectively were found.

In the introduction and validation of the PCR method, we also investigated how long the biological material could be preserved before proper isolation to avoid DNA degradation in unsuitable liquid material and, consequently, decrease in sensitivity of PCR. The urine samples of a healthy person were inoculated with 250 live leptospires for 1 mL and the PCR reaction was realized. The samples were then preserved at fridge temperature (4 °C) and tested repeatedly every 24 h. In the urine preserved for 24 and 48 h, the DNA of pathogenic leptospires was demonstrated using PCR. In samples after 72 h the results were repeatedly negative.

Given that the urine of patient No. 10 had been processed after at least 72 h, the DNA in this aggressive environment, often contaminated by bacteria, it had probably already degraded.

The early diagnostics of infection caused by pathogenic leptospires is important, for two main reasons: for the initiation of effective antibiotic therapy and to prevent the development of the severe complications of acute leptospirosis – hepatorenal failure, cardiac or respiratory insufficiency, aseptic meningitis or haemorrhagic diathesis. The literature suggests increasing cases of the severe form of leptospirosis, Weil's disease, and mortality of up to 10% (ref.²⁰).

The detection of the presence of pathogenic leptospires DNA in the blood of the patient should be made in the first week of the disease when no specific antibodies have yet been released which could destroy the leptospires. From the second week of the clinical disease, it is possible to use urine for PCR diagnostic. It is, however, necessary to ensure its early examination or, if preferred, DNA isolation. In the case of meningeal symptoms, the liquor is examined because leptospires are characterized by affinity for nervous tissue and its presence may be demonstrated here.

From the point of view of appropriate laboratory practice in the pre-analytic phase and prevention of falsely negative results, it is necessary to take biological materials before the start of antibiotic therapy or no more than within 24 h. Leptospires are sensitive to most antibiotics; they disintegrate quickly and are then destroyed by the immune system. The result of the examination can be falsely negative in this case even though leptospirosis is still developing in the organism.

Urine taken for the examination via PCR method should be processed within 24 or not later than within 48 h.

Established real-time PCR for detecting the gene encoding surface lipoprotein LipL32 has the advantage of speed (results of examination in 3 h) compared to the previous PCR method (B 64 I, II a G1, G2 primers). The detection limit was determined in 1 – 5 copies of genome/ 1 mL of liquid biological material, and the cost of the examination is lower (used is only one set of primers and chemicals, against parallel PCR examination using B 64 I, II a G1, G2 primers). The disadvantage of real-time PCR method is that the result of examination

is pathogenic leptospires yes x no, and we know nothing about genomospecies. Mentioned fact was removed with using of sequencing analysis.

CONCLUSIONS

Real-time PCR for detecting the gene encoding the superficial lipoprotein LipL32 in the pre-analytic phase, a suitable, rapid and sufficiently reliable method for the diagnostics of the acute form of leptospirosis.

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CONFLICT OF INTEREST STATEMENT

Author’s conflict of interest disclosure: *None declared*

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