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ORIGINAL PAPER

Application of PCR for Specific Diagnosis of Leptospirosis in Humans in Ukraine

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Abstract

Leptospirosis remains one of the most widespread zoonotic diseases in the world and Ukraine, in particular. Ukrainian clinicians have been faced with early detection of the disease due to the availability of only a serological method for routine diagnostics in Ukraine, namely the microscopic agglutination test (MAT). This paper demonstrates the first results of the complex application of MAT and polymerase chain reaction (PCR) for routine verification of leptospirosis, which were first applied simultaneously in Lviv Oblast of Ukraine in 2016. We examined the sera of 150 patients clinically suspected of leptospirosis, 31 of whom were treated at the Lviv Oblast Clinical Hospital for Infectious Diseases (LOCHID). The application of PCR during the first seven days of the disease allowed increasing the share of confirmed leptospirosis cases by 16,1% in patients that were treated in LOCHID during 2016–2017.

Key words: Leptospirosis, diagnostics, microagglutination test, polymerase chain reaction

Introduction

Leptospirosis is a zoonotic bacterial infection, which the causative agent is *Leptospira* spp. that may infect both wild and domestic animals and humans. Humans contract leptospirosis from animals through exposure to contaminated water or by direct contact with an infected animal. Person to person spread does not occur (Dupouey et al. 2014). In Ukraine, a relatively high incidence rate of leptospirosis has been reported (0.70 per 100,000 population in 2019; 295 cases were recorded) (Centers for Disease Control (CDC) of Ministry of Health (MH) of Ukraine 2019; Tsarenko et al. 2019). Between 2006 and 2019 (15 years), leptospirosis's average incidence rate in Ukraine was 0,94 per 100,000.

According to the World Health Organization (WHO) recommendations, leptospirosis in humans should be diagnosed based on a combination of epidemiological and clinical data with mandatory laboratory tests to confirm the diagnosis (WHO 2003). Physicians often diagnose leptospirosis based on epidemiological and clinical data, with subsequent confirmation based on laboratory tests.

Serology tests with different sensitivity and specificity are most commonly used for leptospirosis diagnostics (Postic et al. 2000; Panwala et al. 2015). Several techniques are often used either together or sequentially to establish a correct diagnosis reliably.

The microscopic agglutination test (MAT) developed in 1918 by Martin and Pettit is considered the gold standard for serodiagnosis of leptospirosis and is still recognized as the reference method of diagnostics in leptospirosis laboratory (WHO 2003). Antibodies for leptospirosis develop between 3–10 days after symptom onset; thus, any serologic test must be interpreted accordingly. Serological testing should be tested with a series of two samples; the first sample collected after the onset of disease and a second convalescent sample 7–10 days after the first.

The MAT is rather complicated for implementation, interpretation, and control (Lucchesi et al. 2004). The accuracy of the MAT is approximately 75–80%; however, this sensitivity and specificity are typically only attained during the third week of the disease and often only have a retrospective value for clinicians and patients (OiE Terrestrial Manual 2018). Furthermore, antibodies to

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different spirochetes may cross-react with *Leptospira,* which causes the results to be less reliable (Postic et al. 2000). Therefore, it is highly recommended to increase the MAT's sensitivity using local isolates rather than reference strains (OiE Terrestrial Manual 2018).

The MAT allows detecting of two classes of antibodies, IgM and IgG, in a single reaction. Since live cultures are used, factors such as age and density of *Leptospira* cultures (as they may influence the agglutination titer), the method is still not standardized (WHO 2003). The viability of live cultures of all *Leptospira interrogans* serovars needed for use as antigens must be constantly maintained by the laboratory staff, which is a laborious and challenging task. Another disadvantage of the method is the late appearance of antibodies in the body of patients with leptospirosis, which often has only historical value. During the last 15 years (2003–2017), 378 patients were discharged from LOCHID with leptospirosis diagnosis. Among them, 272 (71.9%) patients were diagnosed with MAT, the remaining 106 (28,1%) patients were diagnosed based on clinical manifestations only since the MAT result was negative, and no other methods were used.

Considering the above-mentioned drawbacks of MAT, the laboratory network in Ukraine has improved the specific diagnostics of leptospirosis at the early stages of the disease. Such laboratory practices are focused on the detection of the *Leptospira* spp. genetic material in the biological fluids of infected patients (Vasiunets et al. 2019). Here we describe an evaluation of a PCR for detecting a specific part of pathogenic *Leptospira* DNA in patient samples during the early phase of infection (Postic 2000). The implementation of the PCR is divided into two categories. The first one is based on detecting the genes, which are universal for these bacteria (*gryB*, *rrs* and *secY*). The second one is based on the detection of genes inherent for pathogenic *Leptospira* (for example, *lipL21*, *lipL32*, *lipL41*, *ligA* or *ligB*) (Thaipadungpanit et al. 2011). However, both PCR categories do not allow identifying *Leptospira* serogroup that caused the disease. Identification of the pathogen is possible only for the genotype *L. interrogans.*

PCR primers that are based on the *lipL32* gene are the most commonly used in PCR kits that have been developed and evaluated for human sample testing. The presence of amplification inhibitors in clinical samples may lead to false-negative results, especially in specimens that could be contaminated. The quality control of PCR for *Leptospira* detection requires attention to the laboratory facilities conditions, the equipment, the workflow process, and the mandatory use of appropriate control samples (Dragon et al. 1993; OiE Terrestrial Manual 2018). Additionally, strict compliance with the procedure and conditions for the selection, as well as the treatment of clinical specimens for PCR, plays a crucial role in order to receive reliable results. The main objective of our study was to analyze the effectiveness of using the MAT with *Leptospira* and PCR of urine for specific laboratory diagnostics of leptospirosis.

Experimental

Materials and Methods

Patient population. The study subjects were patients with suspected leptospirosis, treated in different Lviv Oblast hospitals from 2016 to 2017. We conducted a retrospective analysis of medical records and studied registration data of 150 patients with suspected leptospirosis, whose samples were tested at the Laboratory of Especially Dangerous Infections of the State Institution Lviv Oblast Laboratory Center of Ministry of Health of Ukraine (Laboratory of EDI of SI LOLC). In case of symptoms similar to leptospirosis and/or epidemiological anamnesis typical for this disease, the patients' biological materials were sent to the Laboratory of EDI of SI LOLC to confirm or deny leptospirosis. Also, the biological material of patients whose symptoms even partially resembled leptospirosis was sent to the laboratory. Thus, doctors of different specialties carried out a diagnostic search so that the diagnosis of leptospirosis was in the list of those that should be excluded to establish the final correct diagnosis.

The MAT procedure. The diagnostic kit of live *Leptospira* containing 13 serogroups (*L. icterohaemorrhagiae*, *L. javanica*, *L. canicola*, *L. autumnalis*, *L. australis*, *L. pomona*, *L. grippotyphosa*, *L. bataviae*, *L. tarassovi*, *L. hebdomadis*, *L. pyrogenes*, *L. ballum*, *L. cynopteri*) was used to perform the MAT. The Laboratory of EDI provided these strains. The MAT assay was performed according to the WHO recommendation and internal standard operation protocol (WHO 2003).

The result was considered as a positive and the endpoint titre of serum, with agglutination score equal to or greater than 2, and no lysis signs and agglutination in control. If the MAT results were positive for a few Leptospira serogroups, the serogroup's agglutination with the highest serum titres was considered the final positive result.

The PCR procedure. We used AmpliSens® *Leptospira*-FRT PCR kit (Russian Federation) for the qualitative detection of 16S RNA of pathogenic *Leptospira* genospecies in the biological fluids (blood and urine) by real-time PCR.

The test was performed according to the manufacturer's instruction using Rotor-Gene 3000/6000 (Corbett Research, Australia). The samples were considered positive if the determined Ct value was less than 32. If

Table I
The diagnosis of patients with suspected leptospirosis.

Method of investigation	Total number of patients with suspected leptospirosis (n = 150)		Positive results by different methods		Total confirmation	
	Number of the samples examined (absolute)	Number of the samples examined (%)	Number of positive samples (absolute)	Number of positive samples (%)	Number of positive samples (absolute)	Number of positive samples (%)
MAT	148	98.66	20	13.33	33	22
PCR	30	20	5	3.33		
MAT + PCR	28	18.66	8	5.33		

the Ct value in a sample was higher than this boundary-value than the sample was equivocal.

Urine for analysis (volume 100 ml) was taken into a sterile container. The sample was centrifuged at 9,000–10,000 g for 10 min, and then approximately 99 ml of the supernatant was discarded. 1 ml of the supernatant was left over the precipitate in a test tube and resuspended respectfully. The suspension was transferred to a new tube and concentrated via centrifugation at 13,000 g for 10 min. 900 µl of the supernatant was discarded, and the remaining pellet and supernatant were used for DNA/RNA isolation. If there was no chance to test material within 24 h after sampling, urine was transferred to a centrifuge tube or an Eppendorf tube. The tube's content was mixed with glycerol (~10% v/v) and frozen. It could be stored at ≤ –16°C for one week or at ≤ –68°C for an extended period (Guidelines to AmpliSens® Leptospira-FRT PCR 2017).

For the DNA/RNA extraction, the RIBO-prep kit (Federal Budget Institute of Science Central Research Institute for Epidemiology, Russian Federation) was used (Guidelines to AmpliSens® Leptospira-FRT PCR 2017).

Data analysis. The data on all patients have been grouped into a single database in Microsoft Excel. The results were statistically processed using descriptive statistics and comparative data analysis. All calculations were performed using the StatSoft's Statistica 8.0 application package by Windows.

Ethical approval. The protocol was reviewed and approved by the Ethical Review Board of the Danylo Halytsky Lviv National Medical University.

Results

During 2016–2017, the biological samples collected from 150 patients suspected (clinically) to have leptospirosis were tested at the Laboratory of EDI by MAT (blood was collected after the 7th day of the disease onset) and PCR assay (urine was collected between the 1st and seventh day of the disease). The diagnosis of leptospirosis was confirmed for 33 patients (using MAT or PCR, or MAT and PCR simultaneously) that were equal to 22.0% of the total number of patients, including three fatal cases. (Table I). The diagnosis of two patients who died during the first week of the disease was confirmed by PCR and MAT (the *Leptospira* lysis was observed in the titer 1:100–1:200) but the diagnosis of the patient died on the seventh day of the disease was confirmed only by MAT (1:800).

A more detailed analysis of the data mentioned above for patients who have been treated in the department of the Lviv Oblast Clinical Hospital for Infectious Diseases (during the specified period) revealed that the number of the confirmed diagnoses of leptospirosis among the suspicious cases was significantly different. From 2016 to 2017, 31 patients were discharged from the Lviv Oblast Clinical Hospital for Infectious Diseases with a diagnosis of leptospirosis. Twenty-six of them (83.87%) were diagnosed based on clinical signs and laboratory tests such as MAT and PCR. Despite the negative results of MAT and PCR, five patients (16.13%) were discharged from the hospital with clinical leptospirosis diagnosis because of undeniable clinical and epidemiological findings. Each of those patients had severe jaundice with acute renal insufficiency and changes of parameters in clinical and biochemical assays that are typical for leptospirosis. These patients also responded well to the prescribed treatment and recovered.

Discussion

For many decades, in Lviv Oblast, sporadic cases of leptospirosis have been recorded as isolated cases that are unrelated to each other. In 2016, within the territory of Lviv Oblast, the incidence rate was equal to 0.56 per 100,000 population; in 2017 it was equal to 0.72 per 100,000 population ($p > 0.05$) (Centers for Disease Control (CDC) of Ministry of Health (MH) of Ukraine 2019).

It is worthwhile to say that the number of confirmed cases of leptospirosis among the total number of

examined patients within the oblast is characterized by a very low percentage of diagnosis confirmation (21.71%). This could be explained by the fact that both the samples from the patients with clinical leptospirosis-like symptoms and the patients with generic symptoms that could be due to leptospirosis were sent for testing. In such cases, the diagnosis of leptospirosis was included on the list of diseases to be excluded from to establish a correct diagnosis. Blood/urine collected not only from patients of infectious hospitals/departments was sent to the Laboratory of EDI, but also from the patients who were treated in intensive care units, therapeutic and surgical departments of multi-specialty hospitals of the Lviv Oblast.

Among the patients from specialized hospitals such as the Lviv Oblast Clinical Hospital for Infectious Diseases, the percentage of confirmed leptospirosis cases was significantly different from the suspected cases of leptospirosis. A large number of patients with other diagnoses are treated in this health care establishment, and their diagnoses may often resemble a clinical picture similar to leptospirosis (that have a non-infectious nature of the disease: mechanical jaundice, acute kidney damage of various etiologies, mushroom poisoning, etc.); however, such diagnoses are excluded at the pre-hospitalization stage. Those diseases that have a clinical picture similar to leptospirosis include viral hepatitis, malaria, hemorrhagic fevers, however, these are excluded in the Lviv Oblast Clinical Hospital for Infectious Diseases during the diagnostic phase. Therefore, the number of selected patients suspected of leptospirosis compared to patients with the final diagnosis of leptospirosis was significantly higher. The percentage of confirmed diagnoses among the above-stated patients by the specific laboratory diagnostic methods such as MAT or PCR was equal to 83.87%.

In Ukraine, the issue of a final diagnosis establishment for patients with a typical clinical picture of leptospirosis has remained a subject for discussion, especially in cases when the specific laboratory diagnostic methods do not provide positive results. In the Lviv Oblast Hospital for Infectious Diseases, the number of such patients was equal to 5 (16.13%). These patients' symptoms comply with leptospirosis diagnostic criteria in Ukraine for likely cases of leptospirosis (Order of MOH of Ukraine N° 905 2015). According to this regulation, there is not a standard diagnostic protocol to consistently determine leptospirosis infection. Any patient who meets the clinical criteria and has a possible epidemiological risk meets the criteria of a probable case of leptospirosis, and any patient who complies with the clinical and laboratory criteria meets the criteria of a confirmed case. In our opinion, this position seems to be biased. As we may see, there is a particular reason to use the term neglected or lost zoonosis concerning leptospirosis (Allan et al. 2015). According to some authors, up to 70% of patients with leptospirosis do not seek medical assistance because the clinical course of the disease is relatively mild or similar to other diseases like acute respiratory infections or mild disorders of the gastrointestinal tract and therefore, patients often are self-treated (Phraisuwan et al. 1999; Ashford et al. 2000; Levett 2001; Guerrier 2013; Tubiana et al. 2013).

Therefore, the disease is not identified, and information about these patients is lost. Furthermore, such neglect concerning leptospirosis takes place in Ukraine because of a lack of laboratory confirmation. Therefore, cases of leptospirosis are excluded from the annual state statistical reports, and therefore, the official leptospirosis incidence is underestimated. In contrast to Ukraine, European countries include all of the patients with diagnosed leptospirosis in their annual reports, regardless of laboratory confirmation (ECDC 2014). A similar position is observed in leptospirosis hyperendemic regions. Thus, within the studies conducted in India during the outbreak of leptospirosis, which included 169 patients, only 15.9% of them had positive MAT results, and 36.6% had positive IgM ELISA, whereas the rest of the patients were diagnosed with leptospirosis based on clinical signs alone (Bharadwaj et al. 2002). Obviously, that in case of an outbreak of infectious disease, it is not necessary to obtain a specific confirmation of all cases of the disease (Morgan et al. 2002; Guillois et al. 2018).

However, India is an endemic zone for different hemorrhagic fevers that are characterized by leptospirosis-like symptoms. Coinfection of leptospirosis and Crimean-Congo hemorrhagic fever or hemorrhagic fever with renal syndrome (CCHF, GF with renal syndrome) is common among humans (Golubić and Markotić 2003; Seifi et al. 2016). Consequently, the lack of research data that would exclude the presence of these diseases or their coexistence did not prevent the authors of this research from confirming that there were patients with leptospirosis diagnosed based on clinical findings during this outbreak.

Researchers promote a similar approach to data reporting for leptospirosis cases (confirmed case/probable case) from other countries endemic for leptospirosis such as Sri Lanka and Malaysia (Agampodi et al. 2011; Wei Leon Tan 2016).

In Ukraine, which is endemic for hemorrhagic fever (CCHF, GF with renal syndrome), there are no test systems (for general use) to verify this group of diseases. This omission increases the risk of errors while establishing the diagnosis for patients with leptospirosis-like symptoms based on clinical and epidemiological data only. The research we have conducted has the following advantages: the investigation of samples collected from all patients was conducted in the same

laboratory using the same protocol, equipment, and identical test systems.

The main disadvantage of this study is its retrospective nature, as well as the short study period (2 years). However, the use of PCR for the routine verification of leptospirosis has been started in the Lviv Oblast only since 2016; therefore, the analysis of obtained data over a more extended period would not be possible.

The first results of the PCR implementation have shown the potential to improve the specific diagnosis of leptospirosis in humans at the early stages of the disease. It increased the number of confirmed cases of leptospirosis among all suspected patients by 3.29% and 16.13% among all the patients who were ultimately discharged with a final diagnosis of leptospirosis. Simultaneously, the MAT is considered the gold standard for the diagnosis because it detects and confirms the serogroup of the pathogen and, respectively, to suspect (predict) cause-and-effect relationships with possible sources of human infection. In our opinion, currently, the combined use of PCR and the MAT could be considered as the most practical combination of specific methods for detecting leptospirosis in humans. For Ukrainian clinicians and epidemiologists, the issue of final diagnosis establishment based on a typical clinical picture of leptospirosis and epidemiological anamnesis has remained a subject for discussion. However, the use of new techniques for specific confirmation of this disease reduces the percentage of such patients.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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