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DEVELOPMENT AND VALIDATION OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) FOR DETECTION OF LEPTOSPIR GENETIC MATERIAL

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Abstract. *Primer systems were designed and tested and the method of loop-mediated isothermal amplification was validated for rapid diagnosis of leptospirosis. In general, five sets of primers were calculated and analyzed in situ and in vitro, which were ordered for synthesis and chosen for the future as the most promising. Indicators of analytical sensitivity and detection limits of the method were established, the specificity of the method in its tests with homologous and close to taxonomic characteristics heterologous samples of genetic material was proved and completed reproducibility of the method was established under the conditions of its staging on various laboratory equipment and with reagents from different manufacturers. The developed technique of loop-mediated amplification for the diagnosis of leptospirosis was tested on field samples and its diagnostic effectiveness was established.*

Key words: DNA, leptospirosis, loop-mediated isothermal amplification, primer system

Leptospirosis is considered one of the most common natural-focal zoonotic infections of wild, domestic animals and humans (Bharti et al., 2003). Clinical leptospirosis is common in dogs that can excrete leptospira with the urine in the absence of signs of disease (Ellis, 2010; Turchenko and Zon, 2018).

Laboratory diagnostics of leptospirosis are based on outdated and complicated diagnostic methods (MAT, microscopy in the dark field). In addition to serological methods of research, methods of molecular biology have recently been used - polymerase chain reaction in the classical version and in real-time. These methods have great practicality in routine work, because bacteriological studies in leptospirosis, particularly the isolation of pure culture from field material, are problematic and time-consuming.

The leptospirosis control system requires today additional express methods of laboratory diagnostics. One such area is the establishment of test systems based on loop-mediated isothermal amplification (LAMP) (Tsuqunori et al., 2000). This method allows to identify the DNA of leptospirosis pathogens more effectively than PCR diagnostics, and does not require special expensive equipment. First of all, LAMP continues at a constant temperature mode, so only any simple device that can maintain a constant temperature is needed to implement it - a thermostat, a water bath, etc. The results can be obtained with the field condition using portable ultraviolet radiation detectors. The main advantage of this method is higher sensitivity and specificity compared to traditional methods of molecular genetic studies.

Today, in Ukraine there is an urgent need for a comprehensive study of the leptospirosis problem, which should consist of seromonitoring, the establishment of serological groups of pathogens that circulate in different regions, and it is also necessary to present new more effective systems for rapid diagnosis of leptospirosis to improve the effectiveness of surveillance for these diseases.

The main purpose is to develop and validate loop-mediated isothermal amplification for the detection of leptospira DNA in clinical material samples.

Material and methods. The PUBMED site was used to generate databases about sequences. Nucleotide sequences were stored in FASTA format. PrimerExplorer V5 (<http://primerexplorer.jp/e>) was used to calculate the appropriate primers. Validation of the isothermal amplification method was carried out according to the standard scheme, which consists of determining the following indicators:

Analytical sensitivity – determine the minimum concentration of the DNA matrix in the sample that the test system can identify.

Specificity – determine the ability to exactly and selectively detect a certain substance in the presence of components that may occur in the DNA matrix (impurities, similar chemical compounds).
Limit of detection – using a DNA sample of known concentration, a value was determined close to analytical sensitivity that the test system can identify and 10 samples of each dilution are tested. The experiment was repeated twice on different days to determine the limit of detection – the minimum concentration of the pathogen, which gives at least 95% of positive results (at least 29 out of 30).

Evaluation of reproducibility – reproduced the method under different conditions, on different equipment and using reagents from different manufacturers. To determine the diagnostic effectiveness, positive and negative field DNA samples were isolated from the dog's urine that were selected during the expedition trips. A total of 1,025 DNA samples were tested.

Results. According to the literature data for the construction of oligonucleotide sequences for conducting PCR, the most promising leptospira genes are *flaB*, *ompL1*, *lipL25*, *ligA*, *ligB*, *lipL32*, *lipL41* та *16S ribosomal RNA (rrs)* (Gentilini et al, 2017; Hsu et al., 2017; Chen et al., 2015; Suwancharoen et al, 2016). These genes are highly conserved within the species *Leptospira interrogans*, allowing them to be used to design specific primers. To do this, the sequences of these genes were loaded from the GenBank database for the next multiple sequence alignment using the BioEdit program to determine the relevant regions. Thus, the base of nucleotide sequences of the genes was formed such as *LipL32* (n = 58), *LipL41* (n = 54), *rrs* (n = 69), *ompL1* (n = 48), *flaB* (n = 47). When processing other genes, it turned out that their length does not comply with the requirements that are applied when developing primers for conducting LAMP.

At the next stage, the selected genes were investigated to search for conservative regions and selection within appropriate sites for calculating primers. Thus, within the *rrs* gene, 4 regions were selected, and *LipL32*, *LipL41*, *ompL1*, *flaB* – three. At the same time, it turned out that reverse primers were impossible to select because of the short distances between such regions in the *flaB* and *ompL* genes.

To calculate primers within *LipL32/LipL41* genes and their sequence was analyzed using the PrimerExplorer V5 online resource (fig. 1).

A panel of positive control DNA samples was manufactured for the investigation. To do this, 14-day leptospira cultures of the diagnostic line (13 serogroups) were cultivated. To increase the purity of the samples, and to get rid of the nutrient medium components, they were washed three times in saline solution by centrifugation and dilution of the precipitate. At each washing step, the presence of live leptospira was monitored by dark-field microscopy. Further, DNA was isolated from the obtained pure leptospira samples by sorbent sorption using the commercial kit of the QIAGEN campaign. The concentration of the obtained DNA was measured on a NanoDrop spectrophotometer, and the obtained data were fixed for further calculations of validation parameters (Table 1).

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1  ATG AAA AAA CTT TCG ATT TTG GCT ATC TCC GTT GCA CTC TTT GCA AGC ATT ACC GCT TGT GGT GCT TTC GGT GGT CTG CCA AGC CTA AAA
91  AGC TCT TTT GTT CTG AGC GAG GAC ACA ATC CCA GGG ACA AAC GAA ACC GTA AAA ACG TTA CTT CCC TAC GGA TCT GTG ATC AAC TAT TAC
181 GGA TAC GTA AAG CCA GGA CAA GCG CCG GAC GGT TTA GTC GAT GGA AAC AAA AAA GCA TAC TAT CTC TAT GTT TGG AIT CCT GCC GTA ATC
271 GCT GAA ATG GGA GTT CGT ATG ATT TCC CCA ACA GGC GAA ATC GGT GAA CCA GGC GAT GGA GAC TTA GTA AGC GAC GCT TTC AAA GCG GCT
361 ACC CCA GAA GAA AAA TCA ATG CCA CAT TGG TTT GAT ACT TGG ATC CGT GTA GAA AGA ATG TCG GCG ATT ATG CCT GAC CAA ATC GCC AAA
    >>.....LipL32_F3.....>>
                                >>.....LipL32_FIP.....>>
                                                >>.....LipL32_FIP.....>>
451 GCT GCG AAA GCA AAA CCC GTT CAA AAA TTG GAC GAT GAT GAT GAT GGT GAC GAT ACT TAT AAA GAA GAG AGA CAC AAT AAG TAC AAC TCT
    <<.....LipL32_BIP.....<<
                                <<.....LipL32_BIP.....<<
                                                <<.....LipL32_B3.....<<
541 CTT ACT AGA ATC AAG ATC CCT AAT CCT CCA AAA TCT TTT GAC GAC CTG AAA AAC ATC GAT ACT AAA AAA CTT TTA GTA AGA GGT CTT TAC
631 AGA ATT TCT TTC ACT ACC TAC AAA CCA GGT GAA GTG AAA GGA TCT TTC GTT GCA TCI GTT GGT CTG CTT TTC CCA CCA GGT ATT CCA GGT
721 GTG AGC CCG CTG ATC CAC TCA AAT CCT GAA GAA TTG CAA AAA CAA GCT ATC GCT GCT GAA GAG TCT TTG AAA AAA GCT GCT TCT GAC GCG
811 ACT AAG TAA

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Figure 1. Selection of primer sequences to develop a method for detecting leptospira genetic material based on LAMP

According to the results of bioinformatic calculations, the following primary systems were selected for synthesis, the list of which consisted of five sets:

Set 1:

Lip32_1_F3 TGTTCGATTCTGCGCTAA
 Lip32_1_B3 GCAGCTTTGGCGATTTGG
 Lip32_1_FIP TAAGTCTCCGTCGCTGGCTC-TCGCTGAAATGGGAGTTTCG
 Lip32_1_BIP AGCGGCTACCCAGAAAGAAAA-AGGCATAATCGCCGACATTC
 Lip32_1_LF TTCGCCTGTTGGGGAAATCATA
 Lip32_1_LB GGTTCGATACTTGGATCCGTGTAGA

Set 2:

Lip32_2_F3 CCAGGGACAAACGAAACCG
 Lip32_2_B3 GCTTACTAAGTCTCCGTCGC
 Lip32_2_FIP TAAACCGTCCGGCGCTTGTCACTTCCCTACGGATCTGTGA
 Lip32_2_BIP TGGATTCTGCGCTAATCGCTGCTCACCATTTCGCCTGTT
 Lip32_2_LF TGGCTTTACGTATCCGTAAATAGTTG
 Lip32_2_LB AATGGGAGTTCTGTATGATTTC

Set 3:

Lip32_3_F3 TGTTCGATTCTGCGCTAA
 Lip32_3_B3 TTTTTCGCTTCGAGCTTTGG
 Lip32_3_FIP CGCTTACTAAGTCTCCGTCGCCCCGCTGAAATGGGAGTTTCG
 Lip32_3_BIP AGCGGCTACCCAGAAAGAAAAAGGCATAATCGCCGACATTC
 Lip32_3_LF TTTTCGCTGTTGGGGAAATCAT
 Lip32_3_LB AATGCCACATTGTTTGATACTTG

Set 4:

Lip32_4_F3 AAGCATACTATCTCTATGTTTGG
 Lip32_4_B3 TTGGTCAGGCATAATCGC
 Lip32_4_FIP GCTTACTAAGTCTCCGTCGCCCCGTAATCGCTGAAATGGGA
 Lip32_4_BIP GACGCTTTCAAAGCGGCTACTTCTTTCTACACGGATCCAAG
 Lip32_4_LF GCCTGTTGGGGAAATCATACG
 Lip32_4_LB CCCAGAAGAAAAATCAATGCCACA

Set 5:

Lip32-5-F3 TCTATGTTTGGATTCTGCC
 Lip 32-5-B3 ATCGTCACCATCATCATC
 Lip 32-5-FIP CGCTTACTAAGTCTCCGTCGCGTAATCGCTGAAATGGGAGT
 Lip 32-5-BIP GCGGCTACCCAGAAAGAAAGCATAATCGCCGACATTCT
 Lip 32-5-LF CTCACCGATTTTCGCTGT
 Lip 32-5-LB TGCCACATTGGTTTGATACTTG

Table 1.

Primary concentration leptospiral DNA after isolation:

No	Serological group of leptospirosis	DNA concentration ng/μl	Purity of DNA (ratio of optical density over wavelength 280/240)
1	<i>L. Icterohaemorrhagiae</i>	4,67	1,96
2	<i>L. Canicola</i>	5,66	1,70
3	<i>L. Pomona</i>	3,46	2,75
4	<i>L. Grippotyphosa</i>	1,87	2,40
5	<i>L. Bataviae</i>	1,99	1,47
6	<i>L. Hebdomadis</i>	3,06	1,88
7	<i>L. Tarassovi</i>	5,03	1,66
8	<i>L. Australis</i>	9,51	1,67
9	<i>L. Autumnalis</i>	6,78	1,41
10	<i>L. Javanica</i>	5,10	1,47
11	<i>L. Ballum</i>	4,63	1,46
12	<i>L. Pirogenes</i>	8,21	1,54
13	<i>L. Cynopteri</i>	5,73	1,52

Each DNA sample was tested for the presence of *LipL32* gene using classical PCR to determine the suitability of control samples for use in experiments. As a result of conducted tests, the presence of *LipL32* gene was confirmed in all 13 samples of leptospira DNA (fig. 2).

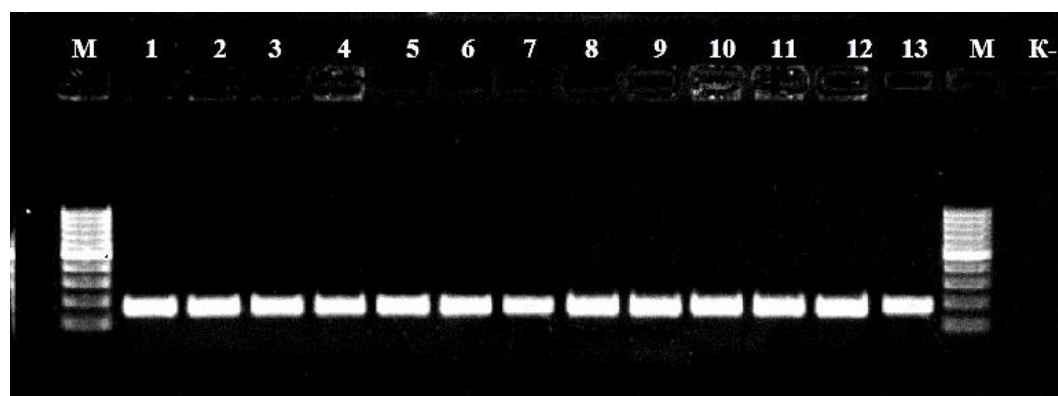


Figure 2. Electropherogram with test results of DNA samples obtained from 13 leptospira diagnostic line for the presence of *LipL32* gene using classical PCR

Notice: M – molecular weight indicator; track 1 - *L. Icterohaemorrhagiae*; track 2 - *L. Canicola*; track 3 - *L. Pomona*; track 4 - *L. Grippityphosa*; track 5 - *L. Bataviae*; track 6 - *L. Hebdomadis*; track 7 - *L. Tarassovi*; track 8 - *L. Australis*; track 9 - *L. Autumnalis*; track 10 - *L. Javanica*; track 11 - *L. Ballum*; track 12 - *L. Pirogenes*; track 13 - *L. Cynopteri*; K - negative control.

Further, different variants of primer systems of five sets were tested separately, according to the standard LAMP protocol, without making additional adjustments. The composition of the reaction mixture for one reaction was as follows: H₂O – 6,75 µl; reaction buffer – 2,5 µL; MgSO₄ – 1 µl; Betaine – 1 µl; dNTPs – 1,5 µl; primers – 6 × (1 µl); Bst polymerase – 1,25 µl. Amplification mode 60 min at 60 ° C.

The first experiment was conducted with *L. Icterohaemorrhagiae* DNA according to the above protocol. Recording of amplification was conducted by electrophoresis in agarose gel, and, in parallel, the presence of fluorescence in ultraviolet light rays of the transilluminator in the reaction tubes themselves was controlled for which the amplification product was stained with ethidium bromide solution. During the test, it was established that amplification occurred with primers of sets №1, №2, №3, №4. With primers of set №5, the formation of amplification products was almost not observed (Figure 3).

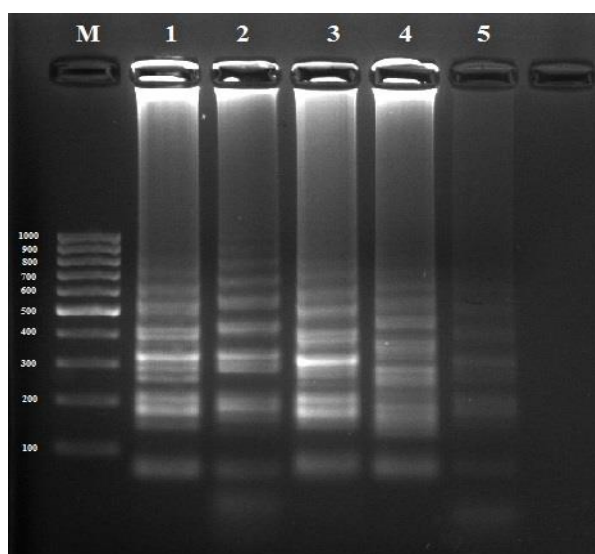


Figure 3. Electropherogram of accounting for loop amplification results with primers of sets 1, 2, 3, 4, 5 (track numbers correspond to set number)

The same result was observed during fluorescence control in the reaction tubes. Fluorescence was ascertained in samples № 1-4 (fig. 4).

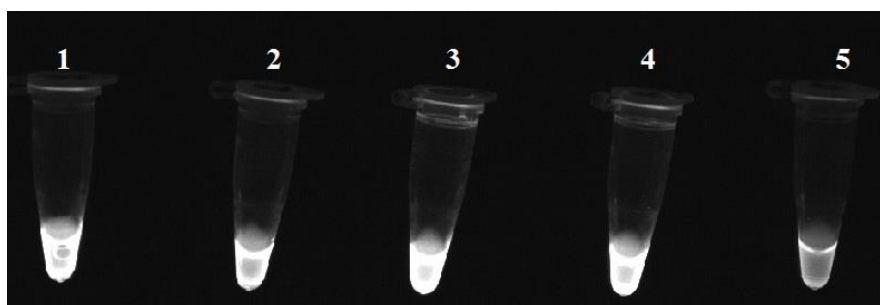


Figure 4. Accounting for loop amplification results by detecting luminescence in UV light with primers of sets 1, 2, 3, 4, 5 (track numbers correspond to the set number)

According to the results of these tests, a primer system from set №3 was chosen for further work, for which a reaction was carried out with control DNA samples of leptospires of the whole diagnostic line. A total of 13 reactions were carried out. Setting tests were made in two stages. At the first stage, leptospira DNA samples with sequence numbers 1-6 were tested, which corresponded to serological groups *L. Icterohaemorrhagiae*, *L. Canicola*, *L. Pomona*, *L. Grippotyphosa*, *L. Bataviae*, *L. Hebdomadis*. Under the second step of the test, LAMP with leptospira DNA samples with sequence numbers 7-13 was investigated, which corresponded to the serological groups *L. Tarassovi*, *L. Australis*, *L. Autumnalis*, *L. Javanica*, *L. Ballum*, *L. Pirogenes*, *L. Cynopteri* (fig. 5).

The presented electropherograms in Figure 4 show that the formation of amplification products was observed with all positive samples of the leptospira diagnostic line.

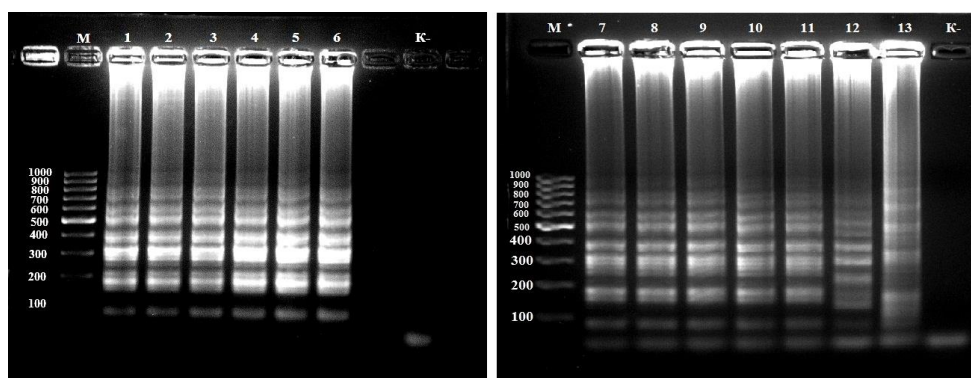


Figure 5. Electropherogram of loop-mediated isothermal amplification results with DNA samples of the 13th leptospira diagnostic line (primers of set №3)

Notice: M - molecular weight indicator; track 1 - *L. Icterohaemorrhagiae*; track 2 - *L. Canicola*; track 3 - *L. Pomona*; track 4 - *L. Grippotyphosa*; track 5 - *L. Bataviae*; track 6 - *L. Hebdomadis*; track 7 - *L. Tarassovi*; track 8 - *L. Australis*; track 9 - *L. Autumnalis*; track 10 - *L. Javanica*; track 11 - *L. Ballum*; track 12 - *L. Pirogenes*; track 13 - *L. Cynopteri*; K - negative control.

Determination of LAMP specificity. To establish the specificity of the method, a panel was made of positive samples of biological material that contained the DNA of pathogens of bacterial infections, which was established during molecular diagnostic investigations with frozen storage at a temperature of -70°C . A part of the panel included five samples, three of which had taxonomic proximity to leptospira and belonged to the same order *Spirochaetales*: causative agents of dysentery (*Brachyspira hyodysenteriae* and *Brachyspira pilosicoli*), whose

DNA was found in pig fecal samples, the pathogen of Lyme disease (*Borrelia burgdorferi*), whose DNA was previously found in dog blood. In addition, the panel included such random pathogens of bacterial infections as *Mycoplasma hyopneumoniae* and *Salmonella Enteritidis*, DNA from which was obtained for the experiment from the cultures of museum strains. Before conducting investigations of the specific method, all control samples were tested for the presence of genetic material of the relevant pathogens using classic PCR. The following primer systems were used for this purpose (Table 2).

Table 2.

Primer systems and sizes of generated PCR products for DNA identification of heterologous infectious pathogens that were used to test the specificity of the method

№	Name of pathogen	Name of primer	PCR-product length (bp)
1	<i>Salmonella Enteritidis</i>	SALM 3	387
		SALM 4	
2	<i>Mycoplasma hyopneumoniae</i>	F1	400-500
		R1	
3	<i>Brachyspira hyodysenteriae</i>	H1	354
		H2	
4	<i>Brachyspira pilosicoli</i>	P1	823
		P2	
5	<i>Borrelia burgdorferi</i>	BOR-com_F	255
		BOR-com_R	

As a result of the tests performed, the presence of appropriate genetic material in the samples for the specificity test was confirmed (fig. 6).

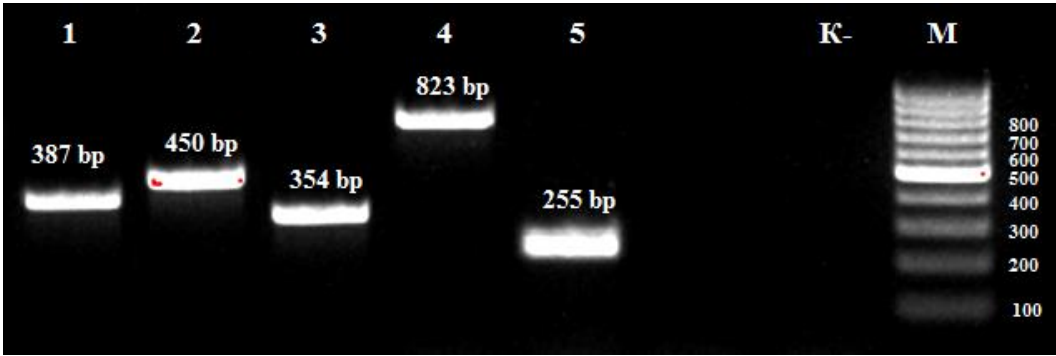


Figure 6. Electropherogram of the results of testing heterologous control samples to determine the specificity of the LAMP method

Notice: Track №1 - *Salmonella Enteritidis*; Track №2 - *Mycoplasma hyopneumoniae*; Track №3 - *Brachyspira hyodysenteriae*; Track №4 - *Brachyspira pilosicoli*; Track №5 - *Borrelia burgdorferi*

The next stage of research was the setting of loop amplification with primers of set №3. Accordingly, as a positive control sample, it was used DNA obtained from *L. Icterohaemorrhagiae* (track № 1), *Salmonella Enteritidis* (track №2) *Mycoplasma hyopneumonia* (track №3), *Brachyspira hyodysenteriae* (track №4) *Brachyspira pilosicoli* (track № 5), *Borrelia burgdorferi* (track № 6). Test system specificity results are presented in electropherogram (fig. 7).

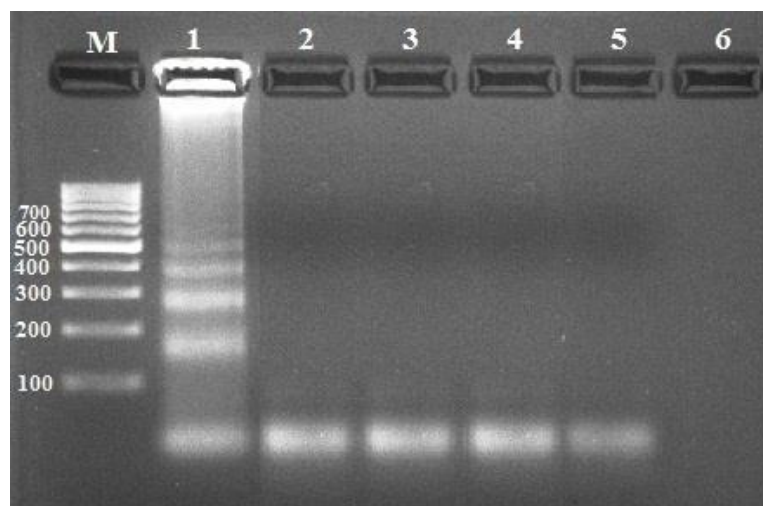


Figure 7. Electropherogram of LAMP results with heterologous control samples

Notice: *L. Icterohaemorrhagiae* (track №1), *Salmonella Enteritidis* (track №2) *Mycoplasma hyopneumonia* (track №3) *Brachyspira hyodysenteriae* (track №4) *Brachyspira pilosicoli* (track №5) *Borrelia burgdorferi* (track №6); M – molecular weight indicator

As can be seen from the presented electropherogram, amplification occurred only in a sample that had leptospira DNA, and other control samples had no amplification.

Determining the sensitivity of the method. A test panel of ten 10-fold dilutions of *L. Icterohaemorrhagiae* DNA. According to the primary concentration determined by the spectrophotometer 4,67 ng/μL, the concentration in the test samples was calculated.

Preparation of samples for conduction amplification was performed in reverse sequence, starting from lowest concentration to highest concentration. Accordingly, track №1 (sample №10); track №2 (sample №9); track №3 (sample №8); track №4 (sample №7); track №5 (sample №6); track №6 (sample №5); track №7 (sample №4); track №8 (sample №3); track №9 (sample №2); track №10 (sample №1), which is shown in Table 3.

Table 3.

Procedure for dilution of control samples and calculated leptospira DNA concentration for sensitivity determination of LAMP method

№	Fold dilution of DNA	DNA concentration
1	1:10	0,467 ng/μL
2	1:100	46,7 pg/μL
3	1:1000	4,67 pg/μL
4	1:10000	0,467 pg/μL
5	1:100000	46,7 fg/μL
6	1:1000000	4,67 fg/μL
7	1:10000000	0,467 fg/μL
8	1:100000000	46,7 ag/μL
9	1:1000000000	4,67 ag/μL
10	1:10000000000	0,467 ag/μL

The results of the system sensitivity test are presented in the electropherogram. As can be seen in Figure 7, the primary weak development of amplification products can be observed in track №4, which corresponded to sample №7 (dilution 1:10000000, DNA concentration 0,467 fg/μL). The first clear production of amplification products was recorded in track №5 (sample №6, dilution 1: 1,000,000, DNA concentration 4,67 fg/μL). This indicator was taken as the analytical sensitivity of the method (fig. 8).

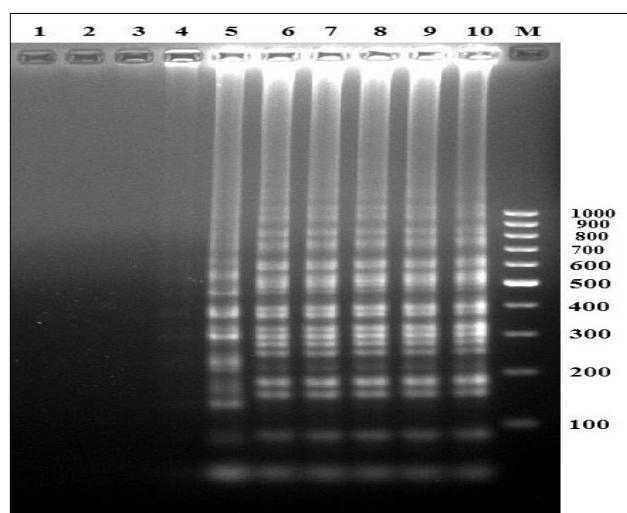


Figure 8. Electropherogram results of the method sensitivity determination

Notice: track №1 (sample №10); track №2 (sample №9); track №3 (sample №8); track №4 (sample №7); track №5 (sample №6); track №6 (sample №5); track №7 (sample №4); track №8 (sample №3); track №9 (sample №2); track №10 (sample №1)

Set the method detection limit. After determining the analytical sensitivity indicators, an experiment was conducted to establish the detection limit. To do this, LAMP was placed with samples having similar values of DNA matrix concentrations to the analytical sensitivity indicator. Samples were collected with concentrations 46.7 fg/μL (dilution 1:100000) and 0.467 pg/μL (dilution 1:10000). After testing 10 samples in three replicates of each dilution, the limit of detection of the test system was set at 46.7 fg/μL (dilution 1:100,000) - 100% of the control samples showed a positive result.

Also, the method showed complete reproducibility when tested with different reagents (three sets of reagents manufactured in China, USA and Germany were used). In addition, various amplification devices were used: BioMetra amplifier, Tertsyk, open solid state thermostat and water bath. The method was reproduced in all tests.

Conclusions. Control of leptospirosis requires the development of new effective means of laboratory rapid diagnosis. Today, the main tool of specialized laboratories is serological studies in microagglutination reactions, for which it is necessary to have live antigens and appropriate conditions. We have proposed and validated a method for rapid diagnosis of leptospirosis based on loop-mediated isothermal amplification (LAMP), as a result of which the most effective primer systems of set №3 were selected, also the analytical sensitivity of the method was determined, which showed the ability to detect leptospira DNA at a concentration of 4.67 fg/μL. The detection limit of the method was determined at 0.467 pg/μL. The specificity of the method has been proved in relation only to the genetic material of pathogenic leptospirs. Full reproducibility is established using reagents for LAMP from different manufacturers and reaction staging on different types of equipment.

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РОЗРОБЛЕННЯ ТА ВАЛІДАЦІЯ ПЕТЛЕВОЇ ІЗОТЕРМАЛЬНОЇ АМПЛІФІКАЦІЇ (LAMP) ДЛЯ ДЕТЕКЦІЇ ГЕНЕТИЧНОГО МАТЕРІАЛУ ЛЕПТОСПІР

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Резюме. Сконструйовано та випробувано праймерні системи та проведено валідацію методики ізотермальної петлевої ампліфікації для експрес діагностики лептоспірозу. Загалом розраховано та проаналізовано *in situ* та *in vitro* п'яти сетів праймерів, що були замовлено для синтезу та обрано для подальших найбільш перспективні. Встановлено показники аналітичної чутливості, межі детекції методики, доведено специфічність методики у випробуваннях її з гомологічними та близькими за таксономічними характеристиками гетерологічними зразками генетичного матеріалу, встановлена повна відтворюваність методики за умов її постановки на різному лабораторному обладнанні та з реагентами різних виробників. Розроблена методика петелевої ампліфікації для діагностики лептоспірозу випробувана на польових зразках та встановлена її діагностична ефективність.

Ключові слова: ДНК, лептоспіроз, петлева ізотермальна ампліфікація, система праймерів

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