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# Genotyping method (MLVA) of pathogenic leptospires for monitoring their distribution in ecosystems

**T.M. Tsarenko[1], V.V. Ukhovskyi[2], L.E. Korniienko[3], N.M. Sakhniuk[1], V.U. Kassich[4], A.P. Palii[5]**

[1]*Bila Tserkva National Agrarian University, pl. Soborna 8/1, Bila Tserkva, 09117, Ukraine*
[2]*Institute of Veterinary Medicine of National Academy of Agrarian Sciences, str. Donetska, 30, Kyiv, 03151, Ukraine*
[3]*State Research Institute for Laboratory Diagnostics and Veterinary and Sanitary Expertise, str. Donetska, 30, Kiev, 03151, Ukraine*
[4]*Sumy National Agrarian University, str. Herasym Kondratiev, 160, Sumy, 40021, Ukraine*
[5]*Kharkiv Petro Vasylenko National Technical University of Agriculture, str. Alchevskyh, 44, Kharkiv, 61002, Ukraine.*
*E-mail: paliy.andriy@ukr.net*
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Leptospirosis is an important problem in veterinary medicine in Ukraine and around the world. The risk of human disease and damage from leptospirosis to livestock make it necessary to study it. For epizootic monitoring of the spread of leptospirosis, it is expedient to use molecular methods for typing pathogenic Leptospiros. In the Bila Tserkva National Agrarian University and the Institute of Veterinary Medicine of the National Academy of Sciences of Ukraine, the molecular method of genotyping of pathogenic leptospiros MLVA-VNTR was tested. The genotyping of reference strains of Leptospiros has been carried out and the genotype of the field strain isolated from the vulgar field mouse from the Ternopol region of Ukraine has been determined. The prospects of using the method for monitoring the distribution of various genotypes of leptospira in ecosystems and the potential use of the method for determining the etiological structure of leptospirosis of farm and domestic animals in Ukraine are shown. For the first time, genotyping of control strains of pathogenic leptospires from the Reference Center of Leptospirosis of the Institute of Veterinary Medicine of the NAAS in Kiev and the correspondence of the serological profiles of the strains of pathogenic leptospiros: Serogroup Canicola, Serovar Canicola Strain Hond Utrecht IV and Serogroup Icterohaemorrhagiae Serovar copenhageni Strain M20 (Fiocruz L1-130) genetic profiles of MLVA. For the first time, the field isolates obtained from wild mice in Ukraine was genotyped in MLVA.
**Keywords:** Leptospira; genotyping; MLVA; VNTR; surveillance; ecosystems

## Introduction

Leptospirosis is a zoonosis which suffers many species of animals and human. The causative agent of leptospirosis is the Leptospira bacteria type. There are 12 pathogenic and 4 saprophytic species; among pathogenic species are more than 250 serovars. Sick on leptospirosis may be agricultural animals (pigs, cattle) and pets (dogs, cats), pathogen carriers of leptospirosis are rodents (Adler, 2015). Antigenically related serovars (that is, serovars that belong to the same serogroup) can belong to different species in genomic classification. DNA-DNA hybridization (DDH) and 16S rRNA phylogenetic analyses have divided the genus Leptospira into three distinct clades (Pathogens, Intermediates and Saprophytes) comprising 22 species (Picardeau, 2017).

According to WHO it was estimated that there are annually 1.03 million clinical cases and 58,900 deaths due to leptospirosis worldwide resulting in 2.90 million DALYs lost each year. Some researchers stress that these figures are underestimated and leptospirosis is a much greater threat to public health than is commonly believed (Schneider et al., 2013).

Leptospirosis is a natural focal disease and some areas are constantly in the danger risk; natural foci of leptospirosis are open self-regulating ecological systems in which populations of focal biomes are in interaction with environmental factors environment, biotic and abiotic. The great danger that leptospirosis causes to humans is due to the emphasis on all aspects of the problem at the ecosystem and social-ecosystemic levels of interaction of the pathogen, animals and humans. The epizootic process among carriers of the pathogenic agent in wildlife occurs entirely at the ecosystem level and the influence on it of the human being is mediated. In connection with the epizootic process there is an epidemiological ecosystem already

on the social-ecosystem, when the epidemic process depends on social characteristics at the local, regional and global levels (Cherkasskiy, 1999). Effective epidemiological surveillance and public health protection is possible in the case of an epidemiological analysis: at the subcellular level, the establishment of the molecular genetic characteristics of the circulating strains of the pathogen, at the cellular level, the analysis of the morphological features of the pathogens, on the organism level, the analysis of the disease data, on ecosystem level - monitoring the etiological structure of the disease, the development of the epizootic process, and at the social-systemic level, the introduction of statistical, sociological and organizing monitoring (Cherkasskiy, 2002).

Analysis of long-term observations (Vasylieva & Kravchuk, 2016) is evidenced by the connection between the circulation of various strain of leptospiros in populations of wild rodents, farm animals and humans. Although there are certain peculiarities in the dynamics and distribution vectors of the pathogens of leptospirosis, it is indisputable that there is a need for continuous monitoring of the prevalence of the pathogens of leptospirosis among wild rodents and farm animals (Zinsstag et al., 2015). The "One Health" concept begins with an awareness of the important opportunities that exist for public health through policies aimed at preventing and controlling the pathogens present in animal populations that are on the verge of humans, animals and the environment. The control of zoonotic pathogens in their animal sources is the most effective and economical way of protecting people. The One Health approach to controlling leptospirosis is important because human infection almost always has a direct impact on animals or on contaminated environments (Petrakovsky & Antonuci, 2018).

Therefore, there is a need for continuous monitoring of epizootic situation of leptospirosis and effective forecasting and risk of disease animals of anti-epizootic measures (Caimi et al., 2017).

The entire territory of Ukraine is endemic with regards to leptospirosis. In Ukraine, according to the Ministry of Health of Ukraine each year reporting 400-500 incidence of leptospirosis cases with 40-60 registered death.

Leptospirosis has big economic importance; the incidence of leptospirosis inflicts substantial economic losses to livestock. Therefore, it is important to control the spread of the causative agent of leptospirosis in the trade, including cross-border trade of live animals (Ukhovskyi et al., 2015).

Another factor that affects public health is leptospirosis of pets, especially dogs, therefore, monitoring of leptospirosis of dogs is necessary from the perspective of the One Health concept (Schuller et al., 2015).

For the validation of the diagnosis of animal leptospirosis in Ukraine normally used a Microscopic agglutination test (MAT). Although this method is recommended according OIE Manual of Standards for Diagnostic Tests and Vaccines and current National Regulations (Guidance on laboratory diagnosis of leptospirosis in Ukraine), this method has a limit.

MAT provides an opportunity to study the antibodies to Leptospira on serogroup level and method show a large number of cross-reactions and accuracy of MAT considerably depend on the animal status during sampling, or an acute phase of the disease, or chronic or carrier. (Adler, 2015). In the veterinary laboratories of Ukraine, a maximum of 17 serovars MAT is carried out (Icterohaemorrhagiae, Canicola, Grippotyphosa, Pomona, Tarassovi, Kabura, Polonica, Mini, Bataviae, Javanica, Ballum, Pyrogenes, Cynopteri, Autumnalis, Australis, Hardjo, Bratislava) but mostly 8 serovars are used (Icterohaemorrhagiae, Canicola, Grippotyphosa, Pomona, Tarassovi, Kabura, Polonica, Bratislava). The MAT method satisfies the needs for the diagnosis of a patient, a person or an animal and the faint suspicion of leptospirosis in the herd of productive animals and the population of domestic animals, but the selection and typing of the causative agent of leptospirosis in order to establish its belonging to a specific stereotype is extremely difficult, labor-intensive, takes a long time and is carried out in several specialized laboratories in the world (Marquez et al., 2017).

The serologic characteristic has a diagnostic and epidemiological significance; now more than 250 pathogenic serovars, grouped in 25 serogroups of the genus Leptospira, are known for serological affinity, but serogroups do not have an official taxonomic status.

MAT is the best diagnostic method when using a panel of strains that coincide locally circulating serovars. Information on serovars is an important contribution to the development of prevention and control and should be used for vaccine selection and risk assessment. Cross agglutinin absorption test (CAAT) is a standard test to establish serovar status (Marquez et al., 2017). In Ukraine serovar status in CAAT not implemented, and the study of the prevalence and epiological structure of pathogens of leptospirosis among the productive (Ukhovskyi et al., 2015) and wild (Pyskun et al., 2018) animals based on serological research in MAT using referential strains, a list of which was given earlier. This practice does not completely satisfy the research needs in the study of the prevalence of pathogenic leptospiros, as the MAT is a serogroup-specific assay and cannot be relied upon to detect the infecting serovar (Marquez et al., 2017).

Over the past decades, methods for genotyping pathogenic leptospires have been developed based on PCR and sequencing of the genome. In particular, the technique of whole genome sequencing brought in valuable information about the genotype of leptospiros, Comparative genomics revealed significant genomic plasticity but labor-intensive and cost of this method limited its use for epidemiological research (Xu et al., 2016).

Method of Multilocus sequence genotyping or multilocus sequence typing (MLST), what is also uses the technique of sequencing part of the genome, according to a definite scheme, looked the most promising for the study of genotypes of pathogenic leptospiros. In order to avoid confusion and unite the efforts of the research groups, an open, accessible website, with a collection of MLST schemes was created, where currently available information on 1278 isolates (http://Leptospira.mlst.net).

As the more High-Resolution method is considered Multi-spacer typing (MST). This method was developed for genotyping strains from the genomic species *Leptospira interrogans* and has been used to identify dominant serovars in France, specifically, Icterohaemorrhagiae, Australis, Canicola and Grippotyphosa (Zilber et al., 2014).

The method that does not require sequenced part of a genome and is performed on the conventional PCR base with electrophoretic detection – it is Multiple-locus variable number of tandem repeati analysis (MLVA), it is a simple and rapid method for categorization of strains and isolates. However, it requires knowledge of variable number of tandem repeats (VNTR) present on the genomes of the microorganisms under investigation, this method is useful in the molecular epidemiological study of Leptospira spp. (Salaün et al., 2006). The MLVA method is widely used in the world for epidemiological studies (Koizumi et al., 2015; Rezasoltani et al., 2015; Colombo et al., 2018).

The purpose of our study was to investigate the value of the MLVA-genotyping method of pathogenic Leptospiras for studding leptospirosis in ecosystems, in particular for the detection of types of leptospiros that are clustered in a carrier population and susceptible animals.

## Materials and methodology

The research was carried out in Research laboratory of novel methods of Bila Tserkva National Agrarian University. Used equipment to perform the classic PCR: Eppendorf MinaSpin ultracentrifuge (USA), a thermoset Thermo-24 Biocom (Russia) and a Vortex Combispin FVL-2400N (Russia) was used to extract DNA by absorption method; thermocycler GeneAmp PCR System 2400 Applied Biosystems (USA) was used for the amplification, for detect amplification results, use the system of horizontal gel electrophoresis Elf-4 (Russia). Photo fixation of the results of electrophoresis was carried out on a digital camera; the explanatory inscriptions on the photo were applied in the Picasa graphical editor.

Specific oligonucleotide primers, which flank fragments of the genome locus of pathogenic Leptospira varies in terms of the number of tandem repeats VNTR-4, -7, -10 specific for *L. interrogans*, *L. kirschneri*, and *L. borgpetersenii* were used. Was used sets of reagents manufactured by Neogen Ltd. (Ukraine) for DNA extraction and amplification by conventional PCR. The amplification products were detected using 2% agar gel electrophoresis (1X TBE-buffer, 135 V, 60 min) with the following identification of the fragment length with a molecular weight marker and compared with the collection of VNTR profiles of the strains described in the literature (Salaün et al., 2006).

For examination from the Laboratory of leptospirosis of Institute of veterinary medicine NAAS (Kyiv) were obtained reference strains pathogenic leptospiros: Serogroup Canicola Serovar Canicola Strain Hond Utrecht IV and Serogroup Icterohaemorrhagiae Serovar Copenhageni Strain M20 (Fiocruz L1-130) and the field strain was isolated from a vulgare field mouse in the Ternopil region of Ukraine. All cultures of Leptospires were delivered into the laboratory in inactive through dissolution 1:1 with a solution for lysis based on guanidine thiocyanate.

## Results and discussion

The MLVA method makes it possible to determine the genotype of pathogenic leptospiros within known collections of genetic profiles (Salaün et al., 2006). Such collections include information about tandem repetitions in different locus of their genome, but the most informative, in our opinion, are VNTR-4, -7, -10 locus, genotyping on which we used in our work.

Regarding the limitations of our research, we did not have enough isolates from other animal species for better testing methods, and could not use serotyping of isolates for comparative analysis because no laboratory in Ukraine performed Cross agglutinin absorption test (CAAT).

The first step was to investigate in MLVA two reference strains used to for MAT in Laboratory of leptospirosis of Institute of veterinary medicine NAAS (Kyiv). The basis of the MLVA method is to determine the length of the amplicon fragment, which depends on the number of tandem repeats of nucleotides (VNTR) compared to the molecular weight marker after horizontal electrophoresis. A feature of the method is the need for visual recognition of fragments of different lengths in the gel after electrophoresis. Such fragments are often very close and their visual discrimination is complicated. The creation of a collection of amplicons with a significant VNTR profile based on the genotyping of reference strains provided an accurate identification of the length of the fragments in the amplicon of the field strain. Analysis of the electrophotogram of fragments with a predetermined number of nucleotide pairs per locus VNTR-4 (bp, base pairs) has demonstrated sufficient opportunities for the differentiation of the number of tandem repetitions of the amplicon (Figure 1).

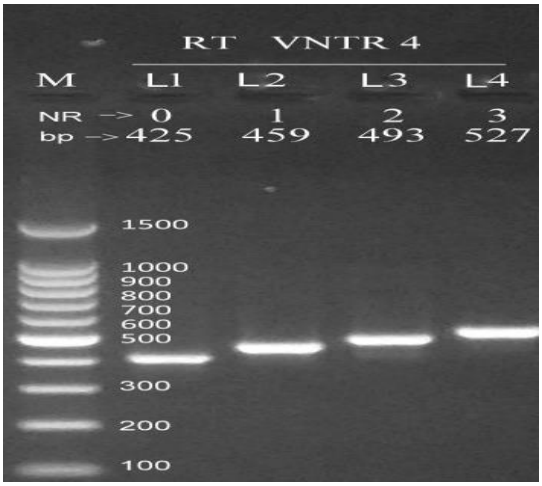

**Figure 1.** Demonstration of the possibility of using the MLVA-VNTR method, for detecting the number of repetitions from 0 to 3 VNTR-4 locus (based on analysis of control strains). M is a band of molecular marker in the range of 1500-100 bp., Control strains L1-L4 Leptospira with a known number of tandem repeats in the VNTR-4 locus, NR is the number of tandem repeats, bp is the number of base pairs.

Reference strains L. Canicola strain Hond Utrecht IV та L. copenhageni Strain M20 (Fiocruz L1-130) showed the full compliance of the MLVA profiles with the ones in the collection on which we were guided by our research (Salaün et al., 2006). The genotyping of reference strains of leptospiros by the MLVA method was the basis of approbation of the method and verification of the results of genotyping.

An isolate of Leptospira obtained from a vulgare field mouse in Ternopil region of Ukraine was analyzed by MLVA method. Its identity as strain Fiocruz L1-130 that is described in the literature as a serovar Copengageni serogroup Icterohaemorrhagiae. Its MLVA profile (Table 1) was identification as same with profile reference strain of Leptospira M20 serovar Copengageni serogroup Icterohaemorrhagiae from the NAAS IVM collection (Figure 2).

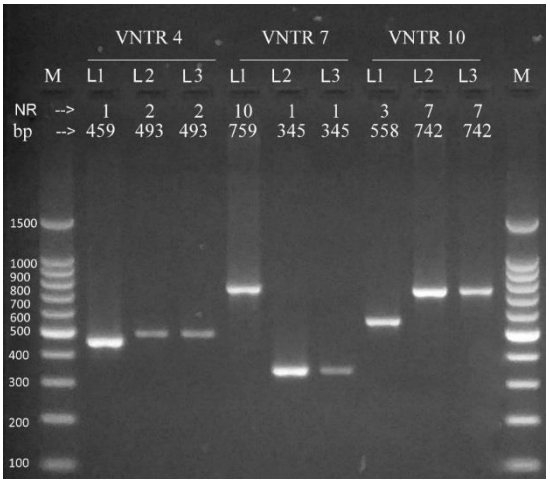


**Figure 2.** Amplicon of the locus VNTR-4, -7, -10 strains L. Hond Utrecht (L1); L. Fiocruz L1-130 (L2) and field isolate Leptospira spp. from a rat in Ukraine (L3) electrophoresis results. Legend: M - band of molecular marker in the range 1500-100 bp., 1, 2, 3 - isolates by VNTR-4, -7, -10, NR - number of tandem repeats, bp - number of base pairs.

**Table 1.** Characteristics of MLVA profiles of examination isolates.

| **Isolate** | **VNTR-4** | | **VNTR-7** | | **VNTR-10** | |
|---|---|---|---|---|---|---|
| | **number of tandem repeats** | **bp** | **number of tandem repeats** | **bp** | **number of tandem repeats** | **bp** |
| L. Hond Utrecht (reference strain) | 1 | 459 | 10 | 759 | 3 | 558 |
| L. Fiocruz L1-130 (reference strain) | 2 | 493 | 1 | 345 | 7 | 742 |
| Field strain of Leptospira (Ukraine) | 2 | 493 | 1 | 345 | 7 | 742 |

The obtained data confirm the possibility of using the MLVA genotyping method to study the distribution of different genotypes of Leptospira. Similar approaches to the establishment of genotypes of pathogenic leptospiros are used by other researchers (Li et al., 2013). The results of our research are consistent with the results of other authors on the effectiveness of the application of the method MLVA-VNTR to determine the genotype of isolates leptospiros and compares the simplicity and low cost of this method (Koizumi et al., 2015; Rezasoltani et al., 2015; Colombo et al., 2018).

It was established that the method of Leptospira molecular genotyping by determining the number of variable tandem repeats of a locus (VNTR-variable number tandem repeats analysis) will be able use for molecular epizootology studies in Ukraine. MLVA method is the simplicity of performance and availability for diagnostic and research laboratories in Ukraine compared to other genotyping methods based on pathogen genome sequencing, in particular Multilocus sequence typing (MLST) or Multispacer Sequence Typing (MST), which need special equipment (Zilber et al., 2014). The research will continue to study the specificities of molecular epizootology of leptospirosis in Ukraine.

The method can be useful for molecular monitoring of pathogens of leptospirosis at the ecosystem level. There is a need for an accurate determination of the type of pathogen that circulates and it is more efficient to do molecular methods based on the conventional PCR laboratory than traditional methods.

## Conclusions

The method of Leptospira molecular genotyping by multilocus analysis of the number of variable tandem repeats (MLVA-VNTR) has been tested in the Research laboratory of novel methods of Bila Tserkva National Agrarian University and in

Laboratory of leptospirosis with the Museum of Microorganisms of Institute of Veterinary Medicine NAAS Ukraine. We shoved possibilities to use MLVA-VNTR method for genotyping pathogenic Leptospires in case with referents strain. The genotype of the reference strain has been correlated with its serological profile. Identification of the genotype of the field isolates pathogenic Leptospira has been completed. The tested method is planned to be implemented in surveillance and control over leptospirosis spreading in Ukraine, and aimed to help with organization control system of leptospirosis area on ecosystem level. Additionally, method of Multiple-Locus Variable number tandem repeat Analysis will be used for molecular epidemiology research in Ukraine. Data of spreading strain of Leptospira can be useful for development and improvement of leptospirosis vaccine formulations.

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