

# INVESTIGATION OF MURINE RODENTS FOR THE PRESENCE OF LEPTOSPIRA DNA BY NESTED PCR

**E. Taseva, I. Christova,  
E. Panayotova, I. Trifonova**

National reference laboratory of vector-borne infections, National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria

## ABSTRACT

Leptospirosis is a zoonanthroponosis with natural foci. People become infected with leptospires either directly from host animals or by means of certain elements of the external environment. Circulation of leptospires in nature is maintained by reservoirs and supporting hosts. For the first time in Bulgaria we applied nested PCR in examining organs of murine rodents for the presence of *Leptospira* spp. DNA. A total of 109 rodents were investigated after being collected from 4 districts in Southern Bulgaria: Plovdiv, Pazardzhik, Smolyan and Blagoevgrad. The genome of *Leptospira* spp. was found in 5 species of rodents. Results show that *Microtus* spp. is a potential carrier of leptospires, especially in urban areas. The high rate of leptospiral DNA-positive rodents captured in the region of Pazardzhik confirms the activity of the epizootic process in this natural focus, where epidemics of benign leptospirosis have been recorded in the past. The introduced method would help to clarify the epidemiological links more quickly in case of a leptospirosis outbreak in some regions. Stronger measures are needed to combat rats, murine rodents and their entry in warehouses,

slaughterhouses and mass caterers, in order to maintain the cleanliness of open-air ponds, water sources and prevent contamination of food products. Future studies in this area would enrich the knowledge on the circulation of leptospires in their reservoirs in more areas of our country.

## KEYWORDS:

*Leptospira* spp., rodents, nested PCR

## INTRODUCTION

Leptospirosis is one of the most widespread bacterial zoonoses in the world. It is caused by over 200 different serovars belonging to several serogroups of the genus *Leptospira* (1). Clinical presentation ranges from mild, flu-like illness to severe symptomatology, including Weil's syndrome with multiple organ failure and often fatal pulmonary haemorrhagic syndrome (13). Contact with stray animals, rodents, poor sanitation, heavy rainfall and flooding are the main risk factors in developing countries, whereas recreational activities, such as freshwater swimming, fishing or sporting events are associated with clinical leptospirosis in developed countries (7, 12). Rodents are the main animal reservoir in urban settings, with *Rattus norvegicus* primarily involved in pathogenic *Leptospira interrogans* serovar Copenhageni transmission (4, 5). Leptospirosis is reported with high prevalence in the rodent population of major cities in developed countries, such as Baltimore in the USA (18), Tokyo in Japan (5) and Copenhagen in Denmark (6).

Human leptospirosis is not a common infection in countries with moderate climate, where Bulgaria also falls, and is therefore often difficult to recognise and treat. A study on pathogenic *Leptospira* in the main reservoirs (rats and mice) would contribute to the faster eradication of the causative agents in their natural outbreaks. In the past, large-scale bacteriological studies have been carried out in Bulgaria with over 1500 animals, mainly rice mice (*Micromys minutus*), water rats (*Arvicola terrestris*), common forest mice (*Apodemus sylvaticus*), yellow-necked mice (*Apodemus flavicollis*) and hedgehogs (*Erinaceus europaeus*) (9).

However, for the last two decades no study has been conducted on detection of leptospires in

## ADDRESS FOR CORRESPONDENCE:

Evgeniya Taseva, PhD  
Department of Microbiology  
National Centre of Infectious and Parasitic Diseases  
44A Gen. N. Stoletov Blvd.  
1233 Sofia, Bulgaria,  
e- mail: evgenia\_taseva@yahoo.com,  
Tel. +359 2832 91 12

rodents. Furthermore, it would be valuable to introduce for the first time a genetic method for detection of leptospiral DNA in rodents.

The purpose of this study is to identify the presence of *Leptospira* spp. DNA in rodent organs for the first time in Bulgaria by using nested PCR.

## MATERIAL AND METHODS

One hundred and nine rodents were tested by a nested PCR method. The specimens were collected from districts in southern Bulgaria for the period December 2012-December 2014. Species of the captured rodents were determined by using the identifier of Ts. Peshev (14). Following dissection of the animals, the spleen was taken and examined for the presence of leptospiral

DNA. Nine of the rodents were newborn and the remaining 100 were adult specimens. Species distribution of rodents and the areas where they were captured are shown in Table 1.

Before examination organs of the rodents were homogenised with Microtube Homogeniser "Bead Bug" with a power of 40 W for 1 minute. *Leptospira* spp. DNA was extracted and purified by DNeasy Blood & Tissue kit (QIAGEN GmbH, Germany). The obtained DNA products were subjected to amplification. Four oligonucleotide primers (BIONEER, Korea) were used with sequences presented in Table 2 (11). DNA extracted from *L. interrogans* serovars Copenhageni, Pomona and Canicola (18-day cultures) were used as a positive control. The amplified products were analysed by electrophoresis on 1.5% agarose gel.

**Table 1.** Distribution of rodents tested by nested PCR by species and district in which they were captured.

District	Pazardjik	Smolyan	Plovdiv	Blagoevgrad	Total	% of the total
<b>Species of rodent</b>						
<i>Apodemus</i> spp.	39	13	1		53	(48.6)
<i>Microtus arvalis</i>	20				20	(18.4)
<i>Microtus</i> spp.	3		2		5	(4.6)
<i>Myodes glareolus</i>	15	3		1	19	(17.4)
<i>Mus musculus</i>	5				5	(4.6)
<i>Mus macedonicus</i>			4		4	(3.7)
<i>Crocidura suaveolens</i>		1			1	(0.9)
<i>Sorex minutus</i>	1				1	(0.9)
<i>Micromys minutes</i>	1				1	(0.9)
Total	84	17	7	1	109	

**Table 2.** Primers used in the enzymatic amplification of the test samples from rodents.

Primers	Nucleotide sequence 5' to 3'	Product in bp
Lepto A	5'-GGCGGCGCGTCTTAAACATG-3'	331 bp
Lepto B	5'-TTCCCCCATTGAGCAAGATT-3'	
Lepto C	5'-CAAGTCAAGCGGAGTAGCAA-3'	289 bp
Lepto D	5'-CTTAACCTGCTGCCTCCCGTA-3'	

## RESULTS

In the wild, besides rats, the main reservoirs of *Leptospira* are the small murine rodents. The relationship between certain species of rodents with a specific serogroup of leptospires is known. In the past, extensive studies have been carried out in Bulgaria on the distribution of leptospires and their reservoirs (9). However, for the last 30 years there is no current data on the spread of leptospires and their reservoirs in

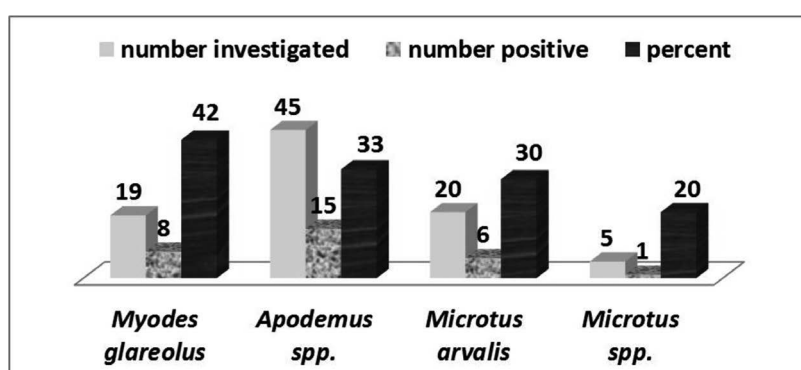
our country.

In our study *Leptospira* spp. DNA was detected in 28.44% (31/109) of the rodents by nested PCR. The genome of *Leptospira* spp. was found in 5 species of rodents: *Apodemus* spp., *Myodes glareolus*, *Microtus arvalis*, *Microtus* spp. – belonging to the order *Rodentia*, and *Sorex minutes* – belonging to the order *Soricomorpha*. They originated from 3 of the surveyed areas: Pazardzhik, Smolyan and Plovdiv (Table 3).

**Table 3.** Distribution of rodents with *Leptospira* spp. DNA.

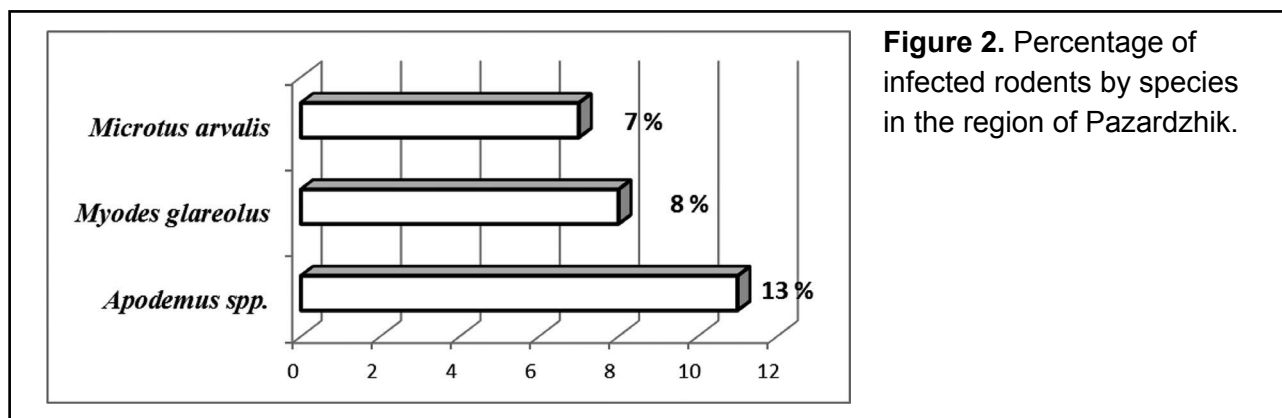
District	Total number of captured rodents	Rodents, carriers of <i>Leptospira</i> spp. (%)	Infected rodents from the total (%)
Pazardzhik	84	25/84 (29.8)	25/109 (22.94)
Smolyan	17	5/17 (29.4)	5/109 (4.59)
Plovdiv	7	1/7 (14.3)	1/109 (0.09)
Blagoevgrad	1	0	0
Total	109	31/109	31/109 (28.44)

Species distribution of rodents with *Leptospira* spp. DNA is presented in Fig. 1. The highest percentage of infected rodents was found for *Myodes glareolus* – 42%, followed by *Apodemus* spp. – 33% and *Microtus arvalis* – 30%. In Pazardzhik was captured 1 *Sorex minutus* which gave a positive result.


**Figure 1.** Species distribution of rodents with *Leptospira* spp. DNA.

The lowest percentage of infected rodents was found for *Microtus* spp. – 20%. The percentage of infected rodents by species in the region of Pazardjik is presented in Fig. 2. The highest

percentage was found for *Apodemus* spp. – 13% (11/84), followed by *Myodes glareolus* – 8% (7/84) and *Microtus arvalis* – 7% (6/84).



**Figure 2.** Percentage of infected rodents by species in the region of Pazardzhik.

The percentage of adult specimens infected with leptospiral DNA was 30% (30/100), and of newborns – 11.11% (1/9). The percentage of infected male rodents was 29.79% (14/47), exceeding that of females – 27.42% (17/62).

## DISCUSSION

In Bulgaria large-scale studies on natural reservoirs of leptospirosis have been conducted in the past (1958-1980) (9, 10). A relatively high proportion of animals positive for leptospirosis was established in Southwestern Bulgaria. This indicates the presence of active natural outbreaks. In the present study, rodents carrying *Leptospira* spp. DNA were found in 3 areas. The highest percentage of infected rodents was found for the region of Pazardzhik – 22.94%. *Leptospira* spp. genome was not detected in the species *Mus musculus* and *Microtus* spp. In Smolyan region 29.41% (5/17) were infected but the number of captured rodents was quite small (only 17). *Leptospira* spp. DNA was present in the species *Apodemus* spp. and *Myodes glareolus*. In Plovdiv region 1 of the captured *Microtus* spp. was infected, whereas *Mus macedonicus* specimens were not infected. In Pazardzhik region the only captured specimen of *Sorex minutus* was infected. The proportion of captured rodents from this species is significantly low – 0.9%, and therefore, it is not statistically significant (Table 1).

The representatives of genus *Apodemus* are found in wetlands in many regions of the country but with low settlement density. Wild murine rodents of the species *Apodemus agrarius* are a reservoir of serogroup Pomona serovar Mozdok (8). In the past, the highest infection rate was found in Petrich (30.1%) and Srebarna Nature Reserve (18.6%). Because of the low settlement density, *Apodemus* species are not capable of sustaining leptospires from this serogroup for long time. Therefore, the infection rate of this species found in our study is comparatively lower – 33% (Fig. 1). The infection rate of *Myodes glareolus* is higher – 42.10%, probably due to the more extensive distribution of this species, especially in wet woodlands. Similar studies have been conducted in a number of European countries. In Croatia was found a high infection rate of rodents from the species *Apodemus* spp. and *Myodes glareolus* with *Leptospira* – 21.5% (16). In Palermo (Italy), the percentage of infected rodents captured near green areas and a small river reached 40%. This was due to the climate change in Sicily from dry and hot to subtropical, with hot wet summer and a sudden storm favouring the spread of pathogenic leptospires (19).

In our country there are 2 species belonging to the family *Soricidae*: *Crocidura leucodon* and *Crocidura suaveolens*. In previous studies, there were positive serological findings of leptospirosis

in 1 out of 267 specimens of *Crocidura suaveolens* (serogroup Icterohaemorrhagiae) (9). No infected rodents of this species were found in the present study. The overall percentage of infected rodents in our study is close to that observed in France – 34.7% (2), in Poland the rates were between 2 and 40% (17), while in Germany – 21% (15). The high rate of infected rodents demonstrated by this study indicates a risk of high level of soil contamination with urine from these animals. The close interaction between humans, animals, water and soil also determines the possibility of outdoor animal infestation. Considering the significantly high percentage of infected *Microtus* spp. rodents – 50% (Fig.1), although only a small number of specimens were captured, it can be concluded that this species is a potential carrier of *Leptospira*, especially in urban areas. Secondary habitats of *Microtus* spp. are farmland and shallow-sloping terrains, and therefore it should be taken into account their movement from dense forests and meadows to urban grasslands and the potential contamination affecting primarily activities such as gardening.

## CONCLUSIONS

The role of small rodents in the epidemiology of leptospirosis is elusive and leaves some aspects unclear. The relationship between reservoirs, people and animals in the epidemiological chain of this infection should be studied further, especially at the molecular biology level. In the future, it would be worthwhile to carry out a more detailed study on detection of *Leptospira* in a larger number of rodents from more areas of the country.

The control of the disease in Bulgaria is limited because our knowledge of the main aspects of epidemiology, including the mode of transmission of *Leptospira* among rat populations, remains incomplete. In our country, after heavy rainfall and floods, there was an increase in the number of cases of leptospirosis in certain regions, which is probably related to an increase in the population of rodents (3). The results we obtained confirm the circulation of *Leptospira* in the reservoirs. The combination of heavy rainfall and the accumulation of waste in deserted buildings and riverbeds are a prerequisite for the

populations of rats and murine rodents to grow in urban areas as well. Further research is needed to provide new perspectives for epidemiological surveillance.

## REFERENCES

- Adler B, de la Pena Moctezuma A. *Leptospira* and leptospirosis. Vet Microbiol. 2010; 140:287-296.
- Aviat F, Blanchard B, Michel V, Blanchet B, Branger C, Hars J, Mansotte F, Brasme L, de Champs C, Bolut P, Mondot P, Faliu J, Rochereau S, Kodjo A, Andre-Fontaine G. *Leptospira* exposure in the human environment in France: A survey in feral rodents and in fresh water. Comp Immunol Microbiol Infect. 2009; 32:463-476.
- Christova I, Tasseva E. *Human leptospirosis in Bulgaria*, 2010-2014. Probl Infect Parasit Dis. 2016; 44(2): 23-29.
- Ciceroni L, Stepan E, Pinto A, Pizzocaro P, Dettori G, Franzin L, et al. *Epidemiological trend of human leptospirosis in Italy between 1994 and 1996*. Eur J Epidemiol. 2000; 16:79-86.
- Koizumi N, Muto M, Tanikawa T, Mizutani H, Sohmura Y, Hayashi E, et al. *Human leptospirosis cases and the prevalence of rats harbouring Leptospira interrogans in urban areas of Tokyo, Japan*. J Med Microbiol. 2009; 58:1227-1230.
- Krojsgaard LH, Villumsen S, Markussen MD, Jensen JS, Leirs H, Heiberg AC. *High prevalence of Leptospira spp. in sewer rats (Rattus norvegicus)*. Epidemiol Infect. 2009; 137:1586-1592.
- Lau CL, Smythe LD, Craig SB, Weinstein P. *Climate change, flooding, urbanization and leptospirosis: fuelling the fire?* Trans R Soc Trop Med Hyg. 2010; 104:631-638.
- Manev Ch. *Distribution and etiological structure of leptospirosis from serogroup Pomona in Bulgaria*. Habilitated Labor, Sofia, 1976; 1-215.
- Manev Ch. *Antigenic characteristics and tanks of leptospires in Bulgaria*. Dissertation for Doctor of science, 1986; Sofia, 1-337.
- Mateev D, Kuyumdzhiev D, Stoyanov D. *Natural reservoirs of leptospires in Petrich and Gotse Delchev. C: Natural outbreaks in Petrich and Gotse Delchev*, 1962; BAS, 33-49.
- Merien F, Amouriauz P, Perolat P, Baranton G and Saint Girons I. *Polymerase chain reaction for detection of Leptospira spp. in clinical samples*. J Clin Microbiol. 1992; 30:2219-2224.
- Mwachui MA, Crump L, Hartskeerl R, Zinsstag J, Hattendorf J. *Environmental and behavioural determinants of leptospirosis transmission: a systematic review*. PLoS Negl Trop Dis. 2015; 9:e0003843.
- Papa A, Theoharidou D, Antoniadis A. *Pulmonary involvement and leptospirosis, Greece*. Emerg Infect Dis. 2009; 15:834-835.
- Peshev T, Peshev D. *Determination of vertebrates in Bulgaria*; 2000.
- Runge M, von Keyserlingk M, Braune S, Becker D, Plenge-Bönig A, Freise JF, Pelz HJ, Esther A. *Distribution of rodenticide resistance and zoonotic pathogens in Norway rats in Lower Saxony and Hamburg, Germany*. Pest Manag Sci. 2012; 69:403-408.
- Tadin A, Tokarz R, Markotić A, Margaletić J, Turk N, Habuš J, Svoboda P, Vucelja M, Desai A, Jain K, Lipkin WI. *Molecular Survey of Zoonotic Agents in Rodents and Other Small Mammals in Croatia*. Am J Trop Med Hyg. 2016; 94(2):466-473.
- Theuerkauf J, Perez J, Taugamo A, Niutoua I, Labrousse D, Gula R, Bogdanowicz W, Jourdan H, Goarant C. *Leptospirosis risk increases with changes in species composition of rat populations*. Naturwissenschaften. 2013; 100:385-388.
- Vinetz JM, Glass GE, Flexner CE, Mueller P, Kaslow DC. *Sporadic urban leptospirosis*. Ann Intern Med. 1996; 125:794-798.
- Vitale M, Di Bella C, Agnello S, Curro V, Vicari D, Vitale F. *Leptospira interrogans survey by PCR in wild rodents coming from different urban areas of Palermo, Italy*. Rev Cubana Med Trop. 2007; 59(1):59-60.