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A small scale survey of Leptospira in mammals from eastern Poland

Angelina Wójcik-Fatla[1], Violetta Zając[1], Jacek Sroka[1,2], Michał Piskorski[3], Ewa Cisak[1], Anna Sawczyn[1], Jacek Dutkiewicz[1]

[1] *Department of Zoonoses, Institute of Rural Health, Lublin, Poland*
[2] *Department of Parasitology, National Veterinary Research Institute, Puławy, Poland*
[3] *Department of Comparative Anatomy and Anthropology, Maria Curie-Skłodowska University, Lublin, Poland*

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Abstract

Samples of 30 dead small mammals each were collected on area 'A' located in eastern Poland and exposed to floods by the Vistula river, and on area 'B', also located in eastern Poland, but not exposed to floods. Kidneys and livers of the mammals were examined by the PCR and nested PCR methods for the presence of *Leptospira* DNA. From 7 species of small mammals examined, the presence of *Leptospira* DNA was detected in 2 of them. The prevalence of positive results was greatest in *Apodemus agrarius* which was the mostly numerous mammal species (14 out of total 39 specimens, 35.9%). The presence of *Leptospira* DNA was also found in *Microtus arvalis* (1 out of 1 specimen, 100%), whereas the remaining 5 species (*Apodemus flavicollis*, *Apodemus sylvaticus, Microtus agrestis, Myodes glareolus, Sorex araneus*) were negative. No significant difference in the prevalence of positive findings was found between the small mammals from areas 'A' exposed to flooding, compared to those from area 'B' not exposed to flooding (20.0% vs. 30.0%, p=0.3748). The overall positivity of the examined small mammals population from areas 'A' and 'B' was 25.0%. The prevalence of dual positivity (leptospiral DNA found both in kidney and liver) was greater in the mammals from areas exposed to flooding compared to those from areas not exposed to flooding (16.7% vs. 6.7%), but this dependence was also not significant (p=0.2382).

Key words

Leptospira, epidemiology, small mammals, PCR, floods, eastern Poland

INTRODUCTION

Leptospirosis is regarded as the most widespread zoonosis in the world and represents a re-emerging health problem because of increasing incidence in humans and domestic animals [1, 2, 3, 4, 5, 6, 7, 8]. The disease is caused by thin, motile spirochetes belonging to the genus *Leptospira*, comprising at least 13 pathogenic and 6 saprophytic species. The bacteria usually survive in the renal tubules of wild and domestic mammals [1, 5]. The main carriers of leptospirae are rodents, which excrete vast quantities of bacteria into the environment, and occupy a central position in the circulation of *Leptospira* in nature [3, 4, 9, 10, 11]. Humans become infected most commonly through occupational, recreational, or domestic contact of skin with the urine of infected animals, either directly or via contaminated water or soil. Depending on the species, the host's immune status, and many other known and unknown factors, the disease can run as a mild, flu-like illness or a severe infection able to cause serious multi-organ or systemic disorders leading to death [1, 2, 3, 4, 12]. With global climate change, extreme weather events such as cyclones and floods are expected to occur with increasing frequency and greater intensity, and may potentially result in an upsurge in the disease incidence as well as the magnitude of leptospirosis outbreaks [4, 5, 12, 13].

The aim of the presented study was to investigate the prevalence of *Leptospira* spp. in small mammals living on the territories of 2 rural communities of the Lublin Province in eastern Poland: community 'A' situated in the western part of the province on the Vistula river and exposed to floods, and community 'B, situated near the central part of the province and not exposed to floods. The study was been carried out within a bigger project on the incidence of *Leptospira* in humans, domestic and wild mammals, ticks, water and soil [11, 14].

Address for correspondence: Angelina Wójcik-Fatla, Institute of Rural Health, Jaczewskiego 2, 20-090 Lublin, Poland
e-mail: afatla@poczta.onet.pl

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MATERIALS AND METHODS

Collection of mammals. Samples of 30 dead small mammals each were collected during the summer/autumn season on area 'A' located in western part of the Lublin Province in eastern Poland, and exposed to floods by the Vistula river, and on area 'B', located in the central part of the province and not exposed to floods. After collection, the mammals were placed in plastic containers and stored at -80°C for further investigation. A total collection of 60 mammals consisted of the following rodent and insectivore species: striped field mouse (*Apodemus agrarius*) – 39 specimens (sp), common shrew (*Sorex araneus*) – 8 sp., bank vole (*Myodes glareolus*) – 5 sp., wood mouse (*Apodemus sylvaticus*) – 5 sp., common vole (*Microtus arvalis*) – 1 sp., field vole (*Microtus agrestis*) – 1 sp., yellow-necked mouse (*Apodemus flavicollis*) – 1 sp.). The organs of the collected small mammals were earlier examined for the presence of hantavirus RNA [15].

No cases of clinical leptospirosis in humans were noted in the areas where small mammals were collected.

DNA isolation. After thawing, the small mammals were subjected to necropsy and the organs (kidney and liver) were placed in the separate vials. Total DNA was extracted from homogenized organs using the QIAamp DNA Mini

Kit (Qiagen, USA), according to the producer's instructions for tissues.

PCR assay. The nested-PCR method was used to identify DNA of *Leptospira* spp.

***Leptospira* spp. DNA identification.** The gene fragment of LipL32 lipoprotein was used as a genetic marker to detect *Leptospira* spp. DNA [16]. Identification was carried out using a pair of primers (Eurogentec, Seraing, Belgium): Amu1 (5'-CGC GCT GCA GTT ACT TAG TCG CGT CAG AAG-3') and Amu2 (5'- CGC GGT CGA CGC TTT CGG TGG TCT GCC AAG c-3') for amplification of the fragment of LipL32 gene. For semi-nested PCR reaction the primers Amu2 and AmuN (5'-CTA TGT TTG GAT TCC TGC-3') were used. First PCR reaction in final volume of 25 µl contained: 0.625 U (0.125 µl) of *Taq* DNA polymerase (Qiagen, USA), 1′ PCR buffer (2.5 µl) containing 15 mM $MgCl_2$ (Qiagen, USA), 2.5 µl 2 mM dNTPs (final concentration 0.2 mM) (Fermentas, Vilnius, Lithuania), 1.25 µl of 10 µM of each Amu1 and Amu2 primers, 14.875 µl nuclease-free water (Applied Biosystems Inc., USA) and 2.5 µl of matrix DNA from the mammals organ isolates. The reaction was performed in C1000 Thermal Cycler (BioRad), and consisted of the initial denaturation (3 min at 94°C) and 35 cycles; each of them included the proper denaturation (30 sec at 94°C), primers annealing (30 sec at 55°C), elongation (60 sec at 72°C), and the final elongation (7 min at 72°C). Electrophoresis was performed in 2% agarose gels in standard conditions. The gels were stained with ethidium bromide and read under UV light. 756 bp-long electrophoresis strips were considered positive. As the positive control, the thermally inactivated suspensions of following strains were used: *Leptospira interrogans* serovar Icterohaemorrhagiae, *Leptospira kirschneri* serovar Grippotyphosa, *Leptospira borgpetersenii* serovar Tarassovi, and *Leptospira interrogans* serovar Pomona. (The strains were obtained by the courtesy of Dr Bernard Wasiński from the National Veterinary Research Institute, Puławy, Poland). The negative control, instead of matrix DNA, was nuclease-free water.

Amu2 and AmuN primers were used in re-amplification. 25 µl of the reaction mixture contained: 1.25 U (0.25 µl) of *Taq* DNA polymerase (Qiagen, USA), 1′ PCR buffer (2.5 µl) (Qiagen, USA), 1.25 µl of 2 mM dNTPs (final concentration 0.1 mM) (Fermentas, Vilnius, Lithuania), 1.25 µl of 10 µM of each primer, 16.0 µl nuclease-free water (Applied Biosystems Inc., USA), and 2.5 µl of matrix DNA from first PCR 10′ diluted in nuclease-free water. The time-temperature profile of the reaction was identical with the previous one, with the exception of the primers annealing which was performed at the temperature 58°C. The reaction products were detected in 2% agarose gels in the standard electrophoresis conditions. After ethidium bromide staining, the strips were read under UV light. The samples with a 574 bp-long strip were considered positive.

RESULTS

The results are presented in Table 1. From the 7 species of small mammals examined, the presence of *Leptospira* DNA was detected in 2 of them. The prevalence of positive results was greatest in *Apodemus agrarius* which was the mostly numerous mammal species (14 out of the total of 39 specimens, 35.9%). The presence of *Leptospira* DNA was also found in *Microtus arvalis* (1 out of 1 specimen, 100%), whereas the remaining 5 species (*Apodemus flavicollis*, *Apodemus sylvaticus, Microtus agrestis, Myodes glareolus, Sorex araneus*) were negative. No significant difference in the prevalence of positive findings was found between the small mammals from area 'A' exposed to flooding, compared to those from area 'B', not exposed to flooding (20.0% vs. 30.0%, p=0.3748). The overall positivity of the examined small mammals population from the areas 'A' and 'B' was 25.0%. The prevalence of dual positivity (leptospiral DNA found both in kidney and liver) was greater in the mammals from areas exposed to flooding, compared to those from areas not exposed to flooding (16.7% vs. 6.7%), but this dependence was also not significant (p=0.2382).

DISCUSSION

Many years ago in Poland, following outbreaks of leptospirosis, small mammals were examined for the presence of *Leptospira*. Most of the studies were performed on the territory of Lower Silesia where outbreaks of the disease had been described since 19th century, and attributed to abundant rainfall and flooding, high temperature and an increased number of small mammals [9, 17]. In 1950, Zwierz et al. [18] isolated in that region leptospirae from 12.1% of examined *Rattus norvegicus* and from 7.1% of *Microtus arvalis*, but not from *Rattus rattus*, *Apodemus agrarius* and *Mus musculus*. In 1974, Konarska [19] examined rodents by the agglutination test and reported a total of 8.1% seropositive findings. The positive reactions were found in *Mus musculus* (2.0%), *Apodemus agrarius* (35.3%), and *Microtus arvalis* (9.9%), but not in *Apodemus sylvaticus*. The reactions were evoked by *Leptospira kirschneri* serovar Grippotyphosa, which was earlier identified as a

Table 1. Prevalence of *Leptospira* in the organs of small mammals found on the territory of the Lublin province (eastern Poland).

Area / Species	"A"	"B"	Total
	Infected/Examined (Percent)	Infected/Examined (Percent)	Infected/Examined (Percent)
Apodemus agrarius	0 K + 1 L + 5 (K+L)/21 = 6/21 (28.6%)	3 K + 3 L + 2 (K+L)/18 = 8/18 (44.4%)	3 K + 4 L + 7 (K+L)/39 = 14/39 (35.9%)
Apodemus flavicollis	Not found	0 K + 0 L + 0 (K+L)/1 = 0/1 (0)	0 K + 0 L + 0 (K+L)/1 = 0/1 (0)
Apodemus sylvaticus	0 K + 0 L + 0 (K+L)/4 = 0/4 (0)	0 K + 0 L + 0 (K+L)/1 = 0/1 (0)	0 K + 0 L + 0 (K+L)/5 = 0/5 (0)
Microtus agrestis	0 K + 0 L + 0 (K+L)/1 = 0/1 (0)	Not found	0 K + 0 L + 0 (K+L)/1 = 0/1 (0)
Microtus arvalis	Not found	0 K + 1 L + 0 (K+L)/1 = 1/1 (100%)	0 K + 1 L + 0 (K+L)/1 = 1/1 (100%)
Myodes glareolus	0 K + 0 L + 0 (K+L)/2 = 0/2 (0)	0 K + 0 L + 0 (K+L)/3 = 0/3 (0)	0 K + 0 L + 0 (K+L)/5 = 0/5 (0)
Sorex araneus	0 K + 0 L + 0 (K+L)/2 = 0/2 (0)	0 K + 0 L + 0 (K+L)/6 = 0/6 (0)	0 K + 0 L + 0 (K+L)/8 = 0/8 (0)
Total	0 K + 1 L + 5 (K+L)/30 = 6/30 (20.0%)	3 K + 4 L + 2 (K+L)/30 = 9/30 (30.0%)	3 K + 5 L + 7 (K+L)/60 = 15/60 (25.0%)

1 K = infected kidney;1 L = infected liver; 1 (K+L) = infected kidney and liver.

cause of human cases of leptospirosis on the territory of Lower Silesia [9, 17].

In 1958, Zwierz et al. [20] carried out an extensive investigation of small mammals from the Tomaszów Lubelski region in southeast Poland after an outbreak of leptospirosis (marsh fever) in that region, by serology, culture and inoculation of animals. They obtained positive results in 15.9% of the examined *Microtus arvalis*, 16% of *Arvicola terrestris*, 45.9% of *Ondatra zibethicus*, 4.5% of *Rattus norvegicus*, 6.3% of *Apodemus sylvaticus*, 4.0% of *Sorex araneus*, 13.0% of *Neomys fodiens*, 10% of *Apodemus agrarius*. No positive reactions were found in *Micromys minutus*, *Talpa europaea*, *Sorex minutus* and *Crocidura leucodon*.

The prevalence of positive results noted in the presented study by PCR is comparable with the above-cited results obtained on the territories of leptospirosis outbreaks by earlier authors using different methods. This seems to indicate that on the areas with the lack not only of a leptospirosis outbreak, but even single cases of this disease [11], the spirochetes may at present circulate within the populations of prone small mammals, in the presented case, *Apodemus agrarius* create a potential risk of disease in humans. As no significant difference in infection rate of rodents was found between the regions exposed and not exposed to floods, it may be assumed that the risk is not limited to the flooded areas.

The total score of positive results recorded for the small mammals examined in the presented study (25.0%) was greater than in some other studies on small mammal reservoir of leptospiroses conducted by serology or PCR in countries with temperate climate: Slovakia [21], Germany [22], Czech Republic [23], Switzerland [24], Japan [25], South Korea [26] and United States [27] (respectively, 5.0%, 7.9%, 12.1%, 12.6%, 11.0%, 12.6%, 10.4%), and similar to the prevalence registered in countries with a warm climate: Sri Lanka [28], Peru [29], Trinidad [30] and Thailand [31] (respectively, 17.5%, 20.3%, 25.6%, 30.0%). Even though the differences in the study methods and small mammals species were taken into consideration, these data seem to indicate that eastern Poland, in spite of the current lack of leptospirosis epidemics, may be regarded as an area of heightened risk of this disease. The presented study also emphasizes the importance of small mammals as a reservoir of *Leptospira* spirochetes.

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