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PREVALENCE OF *FRANCISELLA TULARENSIS* IN THE POPULATION OF SMALL MAMMALS SPECIES IN CONTINENTAL FORESTS OF CROATIA

PREVALENCIJA BAKTERIJE *FRANCISELLA TULARENSIS* U POPULACIJI SITNIH SISAVACA U KONTINENTALNIM ŠUMAMA HRVATSKE

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Summary

Francisella tularensis is a causative agent of tularemia a zoonotic disease that infects wide range of hosts including arthropods, mammals and birds. In this study, the prevalence of tularemia among small mammals in Croatia was investigated. The 444 samples of small rodents and insectivores were collected in eight different localities in continental Croatia during the 2-year study. Spleen samples of: 197 *Apodemus agrarius* (striped field mouse), 78 *Apodemus sylvaticus* (wood mouse), 92 *Apodemus flavicollis* (yellow-necked mouse), 17 *Myodes glareolus* (bank vole), 27 *Mycrotus agrestis* (field vole), 20 *Microtus arvalis* (common vole) and 13 *Sorex araneus* (common shrew) were investigated for the presence of DNA of *Francisella* spp. using qRT-PCR method. Two striped field mice and one wood mouse, originated from the same area - locality of Lipovljani, were found to be positive on *Francisella* spp. revealing the presence of bacteria among small mammals population in Croatia.

KEY WORDS: *Francisella*; Tularemia; DNA; Small rodents; Insectivores; Reservoirs; Endemic areas; Croatia; Prevalence.

INTRODUCTION

UVOD

Francisella tularensis is an etiologic agent of zoonotic disease tularemia. Because of its high infectivity, ease of dissemination, extremely low infectious dose and high fatality rate, the

bacterium has been classified as a potential bioterrorism weapon (Tier 1 select agent) (Kingry and Petersen, 2014). Currently no prophylactic vaccine against tularemia is available. Two subspecies of *F. tularensis* are of clinical importance for humans: *F. tularensis* subsp. *tularensis* (Type A)

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and *F. tularensis* subsp. *holartica* (Type B) (Kingry and Petersen, 2014; Kuehn et al., 2013). They differ in biochemical characteristics, virulence, ecology, epidemiology, as well as geographic distribution (Olsufjev and Meshcheryakova, 1983; Staples et al., 2006). Type A strain occurs mostly in humans and animals in North America (Olsufjev and Meshcheryakova, 1983; Keim et al., 2007). Type B strain is found all over the northern hemisphere, causing disease mainly on European continent. Type B strain is further divided into four main genetic clades including B.4, B.6, B.12 and B.16. (Kuehn et al., 2013) and it is less pathogenic for humans than Type A strains (Olsufjev and Meshcheryakova, 1983; Keim et al., 2007). Infection can be acquired through the skin (bite of an infected vector or direct contact with infected animals), by inhalation of infective agent or by ingestion of contaminated water or food (Kingry and Petersen, 2014). Route of bacterial entry into organism determines the final clinical outcome of disease in humans, which are: ulceroglandular, oculoglandular, oropharyngeal,

gastrointestinal or pneumonic tularemia (Kingry and Petersen, 2014; Maurin and Gyuranecz, 2016; Oyston, 2008; Ellis et al., 2002).

Little is known about ecology of *Francisella* species, and no animal reservoir has been identified yet. Two main life cycles, aquatic and terrestrial, are of epidemiological importance for *F. tularensis* subsp. *holartica* in Europe. The aquatic cycle is present more in south part of Europe including Bulgaria, Kosovo and Turkey, but has been reported in Sweden and Finland as well. Humans are infected through water contaminated with animal excrement. The terrestrial cycle involves small animals and vectors and predominates in countries of Western and Central Europe: Austria, France, Germany, Hungary, Switzerland, Slovakia and Czech Republic (Maurin and Gyuranecz, 2016; Morner, 1992). Rodents, such as meadow voles, water voles, common voles, mice, ground squirrels, beavers, muskrats and lagomorphs like rabbits and hares, in both, terrestrial and aquatic system are supposed to be involved in maintenance



Figure 1. Map of Croatia showing localities of distinct searched areas: Velika Gorica, Lipovljani, Nova Subocka, Stara Gradiška, Sunja, Županja, Koprivnica and Čakovec (Adapted to source: https://d-maps.com/carte.php?num_car=2172&lang=en).

Slika 1. Karta Hrvatske s označenim lokalitetima pretraženih područja: Velika Gorica, Lipovljani, Nova Subocka, Stara Gradiška, Sunja, Županja, Koprivnica i Čakovec (Prilagođeno sa izvora: https://d-maps.com/carte.php?num_car=2172&lang=en).

of the bacterium in the environment. Thereby, these animals could serve as potential reservoirs of bacterium, providing important source of infection for humans (Maurin and Gyuranecz, 2016; Ozanic et al., 2015; Tarnvik, 2007).

In the last twenty years, epizootic screening studies of prevalence of *F. tularensis* among small mammals have been described in different European countries including Hungary (Gyuranecz et al., 2011), Kosovo (Reintjes et al., 2002), Switzerland (Origgi et al., 2015), Slovakia and Austria (Gurycova et al., 2001). Hence, last epizootic reports on prevalence of *F. tularensis* in Croatia date from the middle seventies of the last century (Boric et al., 1976). Recently, Tadin et al. (2016) reported the presence of multiple zoonotic pathogens in rodent species in Croatia with high abundance of *Leptospira* and *Hantaviruses* and low rate of *Francisella tularensis* (0.8 %) infection in *A. agrarius*.

The purpose of this study is to screen for the frequency of *Francisella* spp. among small mammals in distinct areas in Croatia, where the high estincidence of human tularemia was observed. The current study is also part of the program of Croatian Ministry of Agriculture for prevalence of *F. tularensis* among animals (2016). Samples of small rodents and insectivores were trapped during the two years period in eight different localities in continental Croatia: Velika-Gorica, Lipovljani, Nova Subocka, Stara Gradiška, Sunja, Županja, Koprivnica and Čakovec. In this study, three mice were found to be positive for *F. tularensis*.

MATERIALS AND METHODS

MATERIJALI I METODE

During a two-year long survey (2014-2016), small rodents and insectivores were collected in eight different localities

in Croatia: Lipovljani, Nova Subocka, Velika Gorica, Stara-Gradiška, Županja, Sunja, Koprivnica and Čakovec, using snap traps, according to the previously described guidelines (Gannon and Sikes, 2007). Total of 444 animals were trapped, 69 in Lipovljani, 6 in Nova Subocka, 88 in Velika Gorica, 24 in Sunja, 2 in Stara Gradiška, 158 in Županja, 74 in Koprivnica and 23 in Čakovec. Species were morphologically determined. Collected species of small rodent included: *Apodemus agrarius* (197), *Apodemus sylvaticus* (78), *Apodemus flavicollis* (92), *Myodes glareolus* (17), *Microtus agrestis* (27) and *Microtus arvalis* (20). Collected insectivores included one species, *Sorex araneus* (13). Animals were labelled, and spleens were aseptically dissected. The genomic DNA was extracted from spleen using the Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin, USA). The presence of *F. tularensis* in collected samples was confirmed using commercial PCRmax Ltd™ qPCR tests (PCR max, Beacon Road, Staffordshire, UK) for *F. tularensis* succinate dehydrogenase (*sdhA*) gene, according to manufacturer instruction.

RESULTS

REZULTATI

In this study, 444 spleen samples of small rodents and insectivores were tested for the presence of *Francisella* spp., using qRT-PCR method. Dead rodents and insectivores were collected in eight different localities in continental Croatia: Lipovljani, Nova Subocka, Velika Gorica, Sunja, Županja, Stara Gradiška, Koprivnica and Čakovec (Figure 1). Number of collected species in each locality and results of testing are summarized in Table 1. Our results show that from total of 444 animals collected, only 3 mice (0.67%) of

Table 1. The number of different small rodents and insectivora species collected by area. Numbers in brackets represent samples positive on *F. tularensis*.

Tablica 1. Broj skupljenih sitnih glodavaca i kukcojeda po vrsti i lokalitetu. Brojevi u zagradi označavaju uzorke pozitivne na *F. tularensis*.

	Area								Total N=444
	Lipovljani N=69	Velika Gorica N=88	Sunja N=24	Stara Gradiška N=2	Županja N=158	Koprivnica N=74	Nova Subocka N=6	Čakovec N=23	
<i>A. agrarius</i> (striped field mouse)	64 (2)	22 (0)	14 (0)	-	53 (0)	26 (0)	6 (0)	12 (0)	197
<i>A. sylvaticus</i> (wood mouse)	2 (1)	14 (0)	1 (0)	-	46 (0)	12 (0)	-	3 (0)	78
<i>A. flavicollis</i> (yellow-necked mouse)	1 (0)	38 (0)	1 (0)	-	24 (0)	27 (0)	-	1 (0)	92
<i>M. glareolus</i> (bank vole)	2 (0)	6 (0)	-	-	4 (0)	2 (0)	-	3 (0)	17
<i>M. agrestis</i> (field vole)	-	5 (0)	3 (0)	-	19 (0)	-	-	-	27
<i>M. arvalis</i> (common vole)	-	3 (0)	4 (0)	-	3 (0)	7 (0)	-	3 (0)	20
<i>S. araneus</i> (common shrew)	-	-	1 (0)	2 (0)	9 (0)	-	-	1 (0)	13

Apodemus species (*A. agrarius* and *A. sylvaticus*) were positive on *F. tularensis*. All three positive samples originated from the locality of Lipovljani.

DISCUSSION AND CONCLUSIONS RASPRAVA I ZAKLJUČCI

WHO guidelines on tularemia from 2007 highlighted the importance of continuous monitoring of disease occurrence and spread, as well as reports of outbreaks in humans and animal population (Travnik, 2007). The last complex epidemiological and epizootic investigations of tularemia and search for endemic areas in Croatia dates from around 1960s and 1970s, all of the malong Sava Valley. In three reported outbreaks, hares (*Lepus europaeus*), were found to be a source of infection for humans. Hence, in the fourth one, reported in 1974, epizooty of tularemia among small rodents and insectivores was determined as potential source for human infection. This outbreak correlated with peak in field mouse and shrew populations (Borčić et al., 1975). More over, *F. tularensis* was isolated from rodents of *Apodemus* species collected in emerging areas during epizootic research in the mid-960s. This notion and the fact that *Apodemus* species predominated among small rodents population, marked them as main reservoir of *F. tularensis* in investigated period (Heneberg et al., 1967; Borčić et al., 1968).

Recently, Tadin et al. (2016) detected two field mice (*A. agrarius*) positive on *F. tularensis*, trapped in locality of Draganić in central Croatia. However, there is no current information about the real presence of *Francisella* among rodent population in Croatia. The current survey is a part of the program approached by Croatian Government, Ministry of Agriculture, with the aim of systematic monitoring of *F. tularensis* occurrence among animals, including small mammals in Republic of Croatia (2016). Tularemia occurrence was initially associated with "lowland" and "water" ecosystems (Borčić, 1973), therefore, most of the localities included in our study are situated in region of Sava Valley, known as natural marsh area. Sava Valley is known as natural focus for diverse rodent-borne zoonotic pathogens, such are *Leptospira* spp. (Borčić et al., 1982; Tadin et al., 2016), Hantaviruses (Borčić and Puntarić, 1996; Tadin et al., 2016; Tadin et al., 2012) and *Babesia* spp. (Tadin et al., 2012). This region was also mentioned as a natural focus of tularemia in Croatia (Borčić et al., 1976). A common vole, animal of high reproductive capacity, and very sensitive to tularemia infection, was determined as a main carrier of *F. tularensis* and the most important species for the spread of tularemia in mentioned ecosystem (Borčić et al., 1976). However, in this study, the presence of *F. tularensis* has not been demonstrated in any of the examined species of voles.

The reason for this may be a dominance of the mice species within collected samples.

There are substantial differences in number of collected samples, between distinct localities included in our survey. We presume that small number of animals obtained in some areas, as Stara Gradiška and Nova Subocka, is primarily related to a time of the year when the collection was performed. Indeed, during the spring and summer seasons, the population of small rodents and insectivores usually decreases. Next, restricted food sources (forest seed yields) also affects the abundance of their population.

Last reported human tularemia outbreak was described in 1998., when small rodents were presumed as potential source of human infection (Brkić et al., 2005). Although, in recent years, human tularemia in Croatia occurs sporadically, fluctuation in rodent population, leading to the occasional rodent abundance and epizootic outbreaks of tularemia may present high risk for human infection. Three mice positive on *Francisella*, detected in locality of Lipovljani, present significant result, leading to the conclusion that *Francisella* still maintains among small mammals in Croatia. More over, locality of Lipovljani is situated in central part of Sava Valley, revealing that this region might remain natural foci of *Francisella* species in Croatia.

As this study involved only eight small regions in Croatia, there is a need for extended epizootic and investigation studies. Further survey should include larger and diverse geographic areas, asmountain-parts (Lika, Gorski Kotar) and coastal parts of Croatia, aswell as other animals (hares, rabbits) and vectors (ticks, mosquitos).

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SAŽETAK

Francisella tularensis uzročnik je zoonoze tularemije, koja ima široki raspon domaćina, uključujući člankonošce, sisavce i ptice. Dvije podvrste *F. tularensis*, subsp. *tularensis* (Tip A) i subsp. *holartica* (Tip B), opisane su kao najčešći uzročnici bolesti u ljudi (Kingry and Petersen, 2014; Kuehn et al., 2013). Tip A i Tip B razlikuju se po svojim biokemijskim značajkama, patogenosti, ekologiji, epidemiologiji te geografskoj rasprostranjenosti. Rasprostranjenost Tipa A uglavnom je ograničena na sjevernoamerički kontinent, za razliku od Tipa B, koji se javlja duž cijele sjeverne polutke, a ujedno je i najčešći uzročnik tularemije u Europi (Keim et al., 2007; Kuehn et al., 2013). No ekologija i kruženje ove bakterije u prirodi do danas još nisu u potpunosti razjašnjeni. Različiti glodavci (voluharice, miševi, tekunice, bizantski štakori, dabrovi) te dvojzupci (kunić i zec) opisani su kao mogući rezervoari *F.tularensis*. Zadnja epidemiološka i epizootiološka istraživanja tularemije u Hrvatskoj datiraju iz šezdesetih i sedamdesetih godina prošloga stoljeća (Borčić et al., 1975). Stoga je cilj ovoga rada bio prikupiti nove podatke o prevalenciji tularemije u populaciji sitnih sisavaca u Hrvatskoj. Ukupno 444 uzoraka sitnih glodavaca i kukcojeda sakupljeno je u razdoblju od dvije godine na osam različitih loka-

liteta u šumama kontinentalne Hrvatske: Lipovljani, Nova Subocka, Velika Gorica, Stara Gradiška, Županja, Sunja, Koprivnica i Čakovec (Slika 1). Pretraženi su uzorci slezene od: 197 *Apodemus agrarius* (poljski miš), 78 *Apodemus sylvaticus* (šumski miš), 92 *Apodemus flavicollis* (žutogrli miš), 17 *Myodes glareolus* (šumska voluharica), 27 *Mycrotus agrestis* (livadna voluharica), 20 *Microtus arvalis* (poljska voluharica) te 13 *Sorex araneus* (rovka). Uzorcisu pretraženi na prisutnost DNA *Francisella* spp. koristeći qRT-PCR metodu. Dobiveni rezultati pokazuju da su od ukupno 444 prikupljene životinje tri uzorka (0.67%), od kojih dva poljska miša te jedan šumski miš, bili pozitivni su na francizelu (Tablica 1). Sva tri pozitivna uzorka prikupljena su na istom području – lokalitetu Lipovljani.

Tularemija je bolest koja se primarno povezuje s „nizinskim“ i „vodenim“ ekosustavima (Borčić, 1973). Područje duž toka rijeke Save (Posavina) opisuje se kao prirodno žarište tularemije u Hrvatskoj (Borčić et al., 1976), akao glavni rezervoar tularemije na tom području navodi se poljska voluharica (Borčić et al., 1976). Trimiša pozitivna na *F. tularensis* upućuju da je ova bakterija i dalje prisutna u populaciji sitnih glodavaca u Hrvatskoj. Nadalje, svi pozitivni uzorci prikupljeni su na lokalitetu Lipovljani, smještenom u srednjem dijelu toka rijeke Save, stoga ova regija zadržava obilježje prirodnog žarišta tularemije u Hrvatskoj.

KLJUČNE RIJEČI: *Francisella*; tularemija, DNK, sitni glodavci, kukcojedi, rezervoari, endemsko područje, Hrvatska, prevalencija.