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Serologic Survey of Orthopoxvirus Infection Among Rodents in Hungary

Miklós Oldal,[1,2] Tarja Sironen,[3] Heikki Henttonen,[4] Olli Vapalahti,[3,5,6] Mónika Madai,[1,2] Győző Horváth,[2] Bianka Dallos,[1,2] Anna Kutas,[1,2] Fanni Földes,[1,2] Gábor Kemenesi,[1,2] Viktória Németh,[1,2] Krisztián Bányai,[7] and Ferenc Jakab[1,2]

Abstract

As a result of discontinuing vaccination against smallpox after the late 1970s, different orthopoxviruses (OPVs), such as cowpox virus (CPXV), have become a re-emerging healthcare threat among zoonotic pathogens. In Hungary, data on OPV prevalence among its rodent host species have been absent. Here, rodents belonging to four species, *i.e*., striped field mouse (*Apodemus agrarius),* yellow-necked mouse (*A. flavicollis*)*,* wood mouse (*A. sylvaticus*) and bank vole (*Myodes glareolus*), were live trapped at 13 sampling plots on a 149-ha area in the Mecsek Mountains, Hungary, from March to September in 2011 and 2012. Rodent sera were collected and screened for OPV-reactive antibodies with an immunfluorescence assay (IFA). Among the 1587 tested rodents, 286 (18.0%) harbored OPV-specific antibodies. Seroprevalence was the highest for the bank vole (71.4%) and the striped field mouse (66.7%). Due to a masting event in the autumn of 2011 across Central Europe, the abundance of bank voles increased drastically in the 2012 season, raising the overall OPV seroprevalence. We provide the first data on OPV occurrence and seroprevalence in rodents in Hungary. The circulation of OPV in rodents in densely populated areas warrants further studies to elucidate the zoonotic potential of OPV in humans.

Key Words: Cowpox virus—Zoonosis—Serology.

Introduction

Orthopoxvirus (OPV) is a genus in the family Poxviridae, including multiple species isolated from mammals, *i.e*., buffalopox virus, camelpox virus, cowpox virus (CPXV), ectromelia virus (mousepox), monkeypox virus, raccoonpox virus, taterapox virus, volepox virus as well as the infamous variola and vaccinia viruses. In Europe, CPXV is the only known wildlife-borne OPV and is distributed in Europe, through Siberia (Kinnunen et al. 2011), and adjacent areas of northern and Central Asia (Vorou et al. 2008) to South East Asia. CPXV is a zoonotic agent and is considered the most common OPV infection in Europe (Essbauer et al. 2010). Various animal species as well as humans can contract the infection. The clinical manifestations of CPXV are local skin scabs; systemic infections develop most typically in immunocompromised hosts. Descriptions of human infection have increased since the late 1980s, most likely because of the termination of the smallpox vaccinations (Vorou et al. 2008, Duraffour et al. 2013, Shchelkunov 2013).

Rodents are presumably the largest animal reservoir of CPXV. The first serological evidence of rodent hosts for CPXV was established in Russia (Marennikova et al. 1977) in a retrospective study that showed Wistar rats to be the source of CPXV infections of several animals and humans in the Moscow zoo. The primary route of human infection is via bites of rats or other rodent species or by skin contacts with infected domestic animals that carry the pathogen from the original rodent hosts (Marennnikova et al. 1988, Postma et al. 1991, Lewis-Jones et al. 1993).

[1]Virological Research Group, Szentágothai Research Center, University of Pécs, Pécs, Hungary.
[2]Institute of Biology, Faculty of Sciences, University of Pécs, Pécs, Hungary.
[3]Haartman institute and Dept of Veterinary Biosciences, University of Helsinki, Helsinki, Finland.
[4]Finnish Forest Research Institute, Vantaa, Finland.
[5]Hospital District of Helsinki and Uusimaa, Finland.
[6]HUSLAB, HUS, Finland.
[7]Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary.

Studies focusing on wild rodents as reservoirs for CPXV have been conducted in several countries, including United Kingdom, Belgium, Finland, Norway, Sweden, Turkmenia Georgia, and Vietnam (Marennikova et al. 1978, Baxby et al. 1979, Kaplan et al. 1980, Tsanava et al. 1989, Crouch et al. 1995, Boulanger et al. 1996, Tryland et al. 1998, Chantrey et al. 1999, Hazel et al. 2000, Pelkonen et al. 2003, Kinnunen et al. 2011, Forbes et al. 2014). Seroprevalence in the rodents depends on the trapping season and the location, and capture re-capture studies have revealed that seroconversion in rodents in the United Kingdom occurred mainly in late summer and early autumn (Chantrey et al. 1999, Hazel et al. 2000). Bank voles (*Myodes glareolus*), field voles (*Microtus agrestis*), wood mice (*Apodemus sylvaticus*), and rats (*Rattus norvegicus*) are presumably the most significant source of infections. Because isolation and molecular detection of CXPV from rodents is still challenging due to the short viremic period without persistence, serological survey remains the most widely used approach to detect traces of CPXV infections.

In Hungary, the occurrence of OPV infection in the rodents has not been studied so far. As a pilot study, we have tested lung tissue samples from 82 rodents (striped field mouse and yellow-necked mouse) for the presence of CPXV nucleic acid. PCRs were negative, results that were not surprising due to the aforementioned difficulties of CPXV nucleic acid detection. Consequently, we planned the current study as a serological survey. We aimed to determine the reservoir role of various wild rodents and the infection dynamics among these species, using the capture and recapture approach during 2011–2012 in southwestern Hungary. Furthermore, because OPV has not been studied in southeastern Europe, our results could suggest wider implications of this pathogen in this part of Europe.

Materials and Methods

Study area and sampling method

The study region, the forest reserve of Kőszegi-forrás, is located in the Mecsek Mountains (southwestern part of Hungary; Fig. 1) and has a total area of 149 ha. The forest reserve lies on both sides of a deep valley and in a joining plateau, with an elevation of 320–400 meters above sea level. The forest reserve is old beech forest and the surrounding buffer zone is younger oak plantation. Samples were taken both from the core area and the buffer zone.

In 2011 and 2012, 10 and 13 sampling plots were selected, respectively. We used capture–mark–recapture method (CMR), with box-type live traps ($75 \times 95 \times 180$ mm). Bacon and cereals mixed with aniseed extract and vegetable oil were used as bait. Each sampling plot had a grid with six $\times$ six trap stations, 5 meters apart. Monitoring of small mammals was performed in seven trapping periods monthly from March to September in both years. In every month, standard five-night capture occasions were carried out. The traps were checked twice a day at 7 AM and 7 PM. Captured animals were marked by toe tattooing; sex, age, and body mass were also recorded. Blood samples were taken from trapped animals each month using retro-orbital bleeding. In each month during the five-night capture occasion, a blood sample from a given individual animal was only taken once; *i.e.*, only the first monthly capture occasion for a recaptured animal was forwarded for blood sampling. However, in the current study, samples from

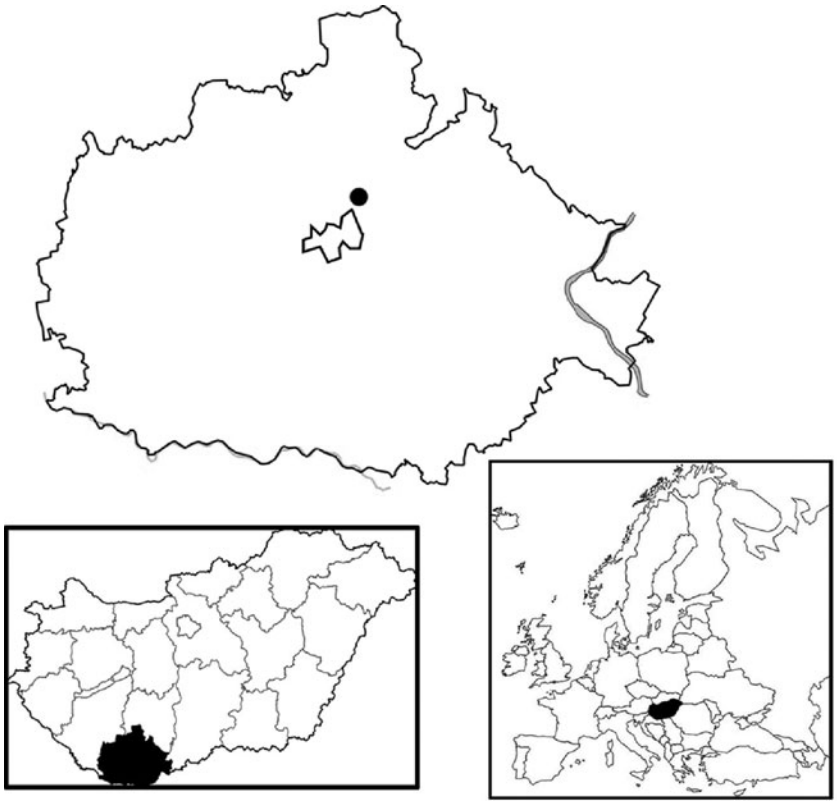

FIG. 1. Location of Kőszegi-forrás forest reserve in Hungary. The forest reserve is represented by a black dot.

recaptured animals were excluded due to the limited number of test slides. Trapped and tested mammals were identified by an expert taxonomist, and all data of the specimens were collected in a computer database (Microsoft Access 2007).

Immunofluorescence assay

OPV-specific immunoglobuliln G (IgG) antibodies were detected from blood samples using immunofluorescent antibody test (IFAT), as described previously (Kallio-Kokko et al. 2006). Briefly, CPXV strain CPXV/FIN/T2000- (Pelkonen et al. 2003) infected Vero cells were detached from cell culture flasks, mixed with uninfected cells, and spotted on slide spots. After air-drying, the cells were fixed with acetone and stored dry at −70°C until use.

After equilibrating of the slides to room temperature, 25 μL of serum sample, diluted in 1:20 in 1 × phosphate-buffered saline (PBS) was added, and the slides were incubated at 37°C for 30 min, followed by three washing steps in PBS for 5 min. A 25-μL amount of goat anti-mouse IgG Alexa Fluor® 488 (Abcam) was used as secondary antibody, applied in 1:1000 dilutions. After three consecutive washing steps, cover slips were added with 50% glycerol, and slides were evaluated with a Nikon Eclipse Ti-U microscope system, at an excitation wavelength of 480 nm.

Statistical analyses

STATISTICA for Windows (data analysis software system), version 8.0, was used to perform chi-squared, Kruskal–Wallis, and Mann–Whitney U-tests. The Kruskal–Wallis test was used to compare seroprevalence among the four rodent species; it was selected due to its availability to test variance in groups with different sample sizes and also extends the analysis to more than two groups. The Mann–Whitney U-test was applied to compare seroprevalence between males and females. It has similar characteristics to the Kruskal–Wallis test, but is only suitable for two groups.

Ethical statement

An ethical statement allowing the trapping, marking, as well as blood sampling of the rodents in the nature reserve area of Kőszegi-forrás was provided by the South-Transdanubian Inspectorate of Environment Protection, Nature and Waters Conservation (Hungary).

Results

With 16,380 trap-nights, we caught a total of 2071 individual rodents from March to September in 2011 and 2012. The density was clearly higher in 2012 than in 2011 (Table 1). Captured animals belonged to four species: 1333 yellow-necked mice (*Apodemus flavicollis*), 539 bank voles, 152 wood mice, and 47 striped field mice (*Apodemus agrarius*). Out of the 2071 small mammals collected, 1587 individuals were screened for OPV antibodies. In total, 286 (18.0%) animals harbored OPV-specific antibodies. The total seroprevalence per species was as follows: 68.3% (215/315) in bank vole, 41.7% (15/36) in striped field mouse, 4.9% (6/122) in wood mouse, and 4.5% (50/1114) in yellow-necked mouse. On the basis of the Kruskal–Wallis test, seropositivity among the bank voles was significantly higher than in the yellow-necked mice or the wood mice ($H[3, n=47]=18.803$, $p=0.0003$). This result was caused by the significantly high abundance of the bank voles compared to the *Apodemus* species (post hoc Dunn test: bank vole versus yellow-necked mouse, $z=3.46$, $p=0.0032$; bank vole versus wood mouse, $z=3.8$, $p=0.0008$).

Sexual distribution of the total tested and seropositive rodents was determined. During the 2-year period, the ratio of

Table 1. Seasonal Distribution and Seroprevalence of Tested Rodents in 2011 and 2012

		Myodes glareolus			Apodemus flavicollis			Apodemus agrarius			Apodemus sylvaticus		
		Tested (n)	*Positive* (n)	%	*Tested* (n)	*Positive* (n)	%	*Tested* (n)	*Positive* (n)	%	*Tested* (n)	*Positive* (n)	%
2011	March	7	0	0.0	40	0	0.0	0	0	0	4	0	0
	April	6	4	66.7	107	1	0.9	1	0	0	10	0	0
	May	6	1	16.7	182	6	3.3	0	0	0	13	0	0
	Jun	1	0	0.0	100	1	1.0	3	0	0	15	0	0
	July	8	8	100.0	58	1	1.7	6	1	16.7	9	0	0
	August	15	9	60.0	19	0	0.0	5	0	0	1	0	0
	September	3	1	33.3	3	0	0.0	0	0	0	0	0	0
	Subtotal	46	23	50.0	509	9	1.8	15	1	6.7	52	0	0
2012	March	7	4	57.1	55	2	3.6	3	3	100	5	1	20
	April	22	17	77.3	66	5	7.6	5	4	66.7	9	1	11.1
	May	20	16	84.2	105	8	7.3	9	6	66.7	9	0	0
	Jun	60	50	80.6	126	12	9.2	3	1	33.3	10	2	20
	July	34	8	21	50	0	0	0	0	0	8	0	0
	August	56	38	65.5	153	14	8.7	0	0	0	21	2	9.5
	September	70	59	85.3	50	0	0	1	0	0	8	0	0
	Subtotal	269	192	71.4	605	41	6.8	21	14	66.7	70	6	8.6
	Total	*315*	*215*	*68.3*	*1114*	*50*	*4.5*	*36*	*15*	*41.7*	*122*	*6*	*4.9*

males to females for the total tested four species was as follows: 536:575 for the yellow-necked mouse, 140:174 for the bank vole, 67:55 for the wood mouse, and 20:16 for the striped field mouse (sex of three individual yellow-necked mice and one bank vole could not be determined). A chi-squared test did not show any significant differences between sex of tested animals ($\chi^2 = 4.778$, $p = 0.687$). Similarly, no significant difference was observed regarding sex among total positives per species: Bank voles, 103:111; yellow-necked mice, 26:24; striped field mice, 9:6; and wood mice, 3:3 ($\chi^2 = 0.946$, $p = 0.996$). We compared seroprevalence among males and females for the yellow-necked mice and the bank voles, as these two species had a high enough number of tested individuals. A Mann–Whitney U-test revealed no significant difference in seropositivity among males and females neither for the yellow-necked mice ($z = 0.243$, $p = 0.808$) or the bank voles ($z = 0.582$, $p = 0.560$).

Tested and seropositive rodents are represented in Table 1. In 2011, the number of tested bank voles and striped field mice both peaked in July and August. The number of tested yellow-necked mice and wood mice was highest in May and June, respectively, with only a few positive yellow-necked mice. In 2011, OPV prevalence closely followed population sizes of only the bank vole, whereas in 2012, the latter phenomenon was also characteristic for the yellow-necked and striped field mice. In 2012, the number of tested bank voles and yellow-necked mice both showed a peak in June; the number of tested bank voles increased further in September. The largest number of tested striped field mice and wood mice were captured in May and August, respectively.

Table 2 summarizes the OPV-seropositive bank voles, distributed by sampling plots and sampling period (months). The data show that in 2011, positive rodents accumulated in three major separated areas, at sampling plots 1 and 8 in July and at sampling plot 9 in August. Next year, OPV seropositivity among bank voles was the highest at sampling plots 1, 9, and 13 in June, whereas most affected plots were 1, 5, and 11 in September. Moreover, in the two peaking months, positive bank voles could be identified from all sampling plots; also in August, only sampling plot 13 was free of infected animals. Hence, infection seems to have spread all across the forest reserve area. Note that in 2012, sampling plots 1–10 remained in the same position and an additional three sites were added (sampling plots 11–13). In summary, in a sharp contrast to 2011, OPV-positive bank voles were identified from all sampling plots throughout the whole 2012 season, whereas the most affected sampling sites were 1, 5, 9, 10, and 13.

Discussion

The current study reports data on OPV antibody prevalence in several rodent species in Hungary. Antibodies against OPV were detected in four rodent species of the subfamilies Murinae and Arvicolinae. The main affected species were the bank vole and the striped field mouse, whereas significantly lower seropositivity was detected in the yellow-necked mouse and the wood mouse. It should be mentioned that IFAT with the CPXV antigen can lead to the detection of cross-reactive antibodies against other OPVs. Although, OPVs other than CPXV are not known in Eurasian wild rodents, we have no molecular evidence for CPXV in Hungary so far. On the basis of the results of the current serologic investigation, we assume the presence of CPXV in

Table 2. Number of Seropositive Bank Voles by Sampling Plots and Months in 2011 and 2012

Sampling plots	*March*		*April*		*May*		*June*		*July*		*August*		*September*	
	2011	*2012*	*2011*	*2012*	*2011*	*2012*	*2011*	*2012*	*2011*	*2012*	*2011*	*2012*	*2011*	*2012*
1	0 (6)	0 (1)	0 (6)	0 (1)	0 (12)	1 (1)	0 (6)	7 (10)	3 (13)	0 (4)	1 (12)	3 (17)	1 (1)	8 (19)
2	0 (3)	0 (0)	1 (11)	0 (1)	1 (17)	2 (4)	0 (9)	2 (7)	0 (7)	1 (6)	0 (5)	4 (9)	0 (0)	3 (17)
3	0 (14)	1 (2)	0 (16)	1 (1)	0 (5)	0 (2)	0 (3)	3 (7)	0 (6)	0 (5)	2 (9)	5 (19)	0 (2)	4 (35)
4	0 (4)	0 (0)	0 (5)	0 (0)	0 (3)	0 (0)	0 (0)	5 (8)	0 (2)	0 (6)	0 (0)	2 (5)	0 (0)	2 (19)
5	0 (2)	0 (0)	0 (1)	0 (0)	0 (3)	1 (1)	0 (2)	2 (5)	0 (3)	1 (1)	0 (0)	2 (7)	0 (1)	13 (23)
6	0 (5)	1 (2)	0 (1)	0 (0)	0 (3)	0 (0)	0 (1)	2 (6)	0 (1)	1 (3)	1 (1)	3 (3)	0 (0)	3 (13)
7	0 (0)	0 (0)	0 (1)	1 (1)	0 (0)	2 (3)	0 (1)	2 (5)	0 (1)	1 (2)	0 (0)	1 (4)	0 (0)	2 (2)
8	0 (10)	0 (0)	1 (7)	1 (6)	0 (13)	1 (4)	0 (9)	3 (6)	4 (8)	0 (3)	1 (5)	5 (11)	0 (0)	2 (19)
9	0 (0)	0 (1)	1 (7)	2 (10)	0 (15)	2 (3)	0 (10)	9 (13)	0 (9)	0 (1)	4 (12)	5 (13)	0 (0)	5 (14)
10	0 (7)	2 (4)	1 (7)	5 (17)	0	4 (9)	0 (0)	4 (7)	1 (1)	1 (18)	0 (2)	5 (24)	0 (1)	4 (33)
11	—	0 (0)	—	2 (4)	—	1 (5)	—	1 (4)	—	1 (4)	—	1 (11)	—	6 (14)
12	—	0 (1)	—	2 (5)	—	1 (6)	—	2 (2)	—	2 (9)	—	2 (13)	—	3 (30)
13	—	0 (0)	—	3 (4)	—	1 (6)	—	8 (19)	—	0 (4)	—	0 (6)	—	4 (17)
Total	*0 (51)*	*4 (11)*	*4 (62)*	*17 (50)*	*1 (71)*	*16 (44)*	*0 (41)*	*50 (99)*	*8 (51)*	*8 (66)*	*9 (46)*	*38 (142)*	*1 (5)*	*59 (255)*

Number of captured rodents is in parentheses. Key:

1–3

3–5

7–9

≥ 10

the region, but further molecular-based studies are needed. In laboratory mice, ectromelia virus (ECTV) is also present in Europe, but no evidence of natural infections exists (Fenner 1994, Kinnunen et al. 2011).

The role of the bank vole as a relevant species in CPXV transmission was also described in England, Germany, and Belgium (Crouch et al. 1995, Hazel et al. 2000, Essbauer et al. 2009). Our data with a maximum OPV seroprevalence of 71.4% in the bank vole is most closely related to the findings of 64% in Belgium (Boulanger et al. 1996) and 72% in England (Hazel et al. 2000). In Finland, Pelkonen et al. (2003) reported large geographic variation with the highest value of 91% in the bank vole. Forbes et al. (2014) also found large geographic and seasonal variation (0–93%) in the field vole *M. agrestis* in Finland. In Germany, seroprevalence in field voles was 32%.

Our data show a rather small seroprevalence for the wood mouse (8.6%). A similar low value was found by Essbauer et al. (2009). The yellow-necked mouse, despite being clearly the most common species at the study area, showed low infection rates with a maximum of 6.8%. In comparison, data from Germany show a maximum value of 25% for yellow-necked mouse, (Essbauer et al. 2009).

Longitudinal data show that the bank vole population increased nearly six-fold from 2011 to 2012, whereas OPV seroprevalence increased by roughly 20% in 2012 (from 50% to 71.4%). The seroprevalence in the yellow-necked mouse increased from 1.8% to 6.8 % (3.7-fold), whereas seroprevalence in the striped field mouse jumped from 6.7% to 66.7% (10-fold). Likewise, although there were no seropositive wood mice in 2011, the seroprevalence reached 8.6% in 2012. However, because the total number of trapped and tested striped field mice and wood mice was low, the role of these species as reservoir hosts of OPV in Hungary remains unclear. Moreover, as the studies in Finland (Pelkonen et al. 2003, Forbes et al. 2014) indicate, the distribution and prevalence of CPXV varies a lot geographically, and therefore exact roles of various rodent species as reservoir and spill-over species are not fully clear. Consequently, more sampling in different parts of Hungary is clearly needed.

The explosive bank vole population growth and the increase of seroprevalence of OPV during 2012 in this and other rodent species require some further explanation. In the autumn of 2011, a masting event occurred widely in Europe, from Germany to Slovenia. The increased amount of food probably resulted in the bank vole peak in the summer of 2012 also in Hungary. However, we do not know why yellow-necked mice did not respond to masting this time. Yellow-necked mice are also considered dominant over bank voles. Thus, it seems reasonable to suppose that a masting-induced increase in the bank vole, the likely single most important reservoir of OPV in the study area, provides an explanation to the drastic increases in seroprevalence in other rodents, which apparently had a minor role in OPV carriage. The gradation of the OPV carrier bank vole likely increased the risk of contact with susceptible focal striped field mouse and wood mouse populations, and could have explained the possible OPV outbreak in striped field mouse during early 2012.

The seasonality of OPV-positive rodents in 2011 corresponds to some studies from the United Kingdom (Feore et al. 1997, Chantrey et al. 1999, Hazel et al. 2000), with an increase in seroprevalence in bank vole and striped field mouse in late summer/early autumn. However, in 2012, seroprevalence in the bank vole and the yellow-necked mouse peaked in June and in the striped field mouse in May, whereas wood mouse had a peak in August. These data suggest a delay in transmission of OPV from one species to the other; moreover, it indicates that the population size of the species does not necessarily show similar seasonality in consecutive years. Forbes et al. (2014) discussed the density dependence of viral infection in rodents. They observed that CPXV antibody prevalence showed a delayed density dependence in spring and direct density dependence in fall. This phenomenon is in accordance with our observations, where the bank vole population reached its peak in September, 2012, during which seroprevalence values were the highest.

The cessation of smallpox vaccination and the increased awareness toward CPXV resulted in a growing number of reported rodent-borne OPV infections in Europe (Postma et al. 1991, Marennnikova et al. 1988, Pelkonen et al. 2003, Shchelkunov 2005, Vorou et al. 2008, Essbauer et al. 2010). Additionally, the virus has a wide host range of susceptible animals (Kinnunen et al. 2011, Shchelkunov 2013). As urbanization is extending, more areas are established where the movement of domestic and wild animals overlap, creating new opportunities for transmission of OPVs, such as CXPV to humans.

Conclusions

In conclusion, this study is the first to provide serologic evidence for the circulation of OPV in wild rodent populations in Hungary. Our data indicate that the primary reservoir of OPV is the bank vole, whereas other rodents studied here may serve as secondary reservoirs. These findings might be an important indicator for further molecular-based studies in the region. Our results also suggested that natural fluctuations of rodent populations, driven, *e.g.*, by masting, can affect rodent species composition, thus shaping the epidemiological and ecological landscape of OPV infections in rodents and, thus, potentially increasing the risks of contact with other susceptible species. Our study adds to the knowledge of OPV in Eastern and southeastern Europe, and indicates the commonness of the virus in several reservoir rodent species. The observations of this study might be alerting because orthopoxvirus vaccination has been globally discontinued since the late 1970s. This has resulted in reduction of protective immunity not only against smallpox virus but also against a variety of other OPVs over time, raising the chance of new OPV cases in humans. It appears to be important for public health to monitor OPV seroprevalence in human population born after cessation of smallpox vaccination with or without risk of contact with wild rodents.

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Author Disclosure Statement

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Address correspondence to:
Ferenc Jakab
Virological Research Group
János Szentágothai Research Center
University of Pécs
Ifjúság út 20
H-7624, Pécs
Hungary

E-mail: jakabf@gamma.ttk.pte.hu