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SHORT COMMUNICATION

Detection of *Toxoplasma gondii*, *Neospora caninum*, and *Encephalitozoon cuniculi* in the brains of common voles (*Microtus arvalis*) and water voles (*Arvicola terrestris*) by gene amplification techniques in western Austria (Vorarlberg)

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Abstract Knowledge about the protozoan parasite fauna in voles (Arvicolinae) in Austria is rather limited, although some of these pathogens play an important role in human medicine and cause zoonoses (e.g., *Toxoplasma gondii* and *Encephalitozoon cuniculi*). Others are of relevance in veterinary medicine and have a negative economic impact (e.g., *Neospora caninum*). Two hundred sixty-eight common voles (*Microtus arvalis*) and 86 water voles (*Arvicola terrestris*) from the most western Austrian province, Vorarlberg, were analyzed with PCR techniques for infections with *T. gondii*, *N. caninum*, and *E. cuniculi*. Brain tissues of two common voles (0.7%) and of four water voles (4.7%) tested positive for *T. gondii*. Furthermore, analysis of four common voles (1.5%) and two water voles (2.3%) generated positive findings for *N. caninum*, and brain tissues of 16 common voles (6%) and six water voles (7%) tested positive for *E. cuniculi*. Accordingly, this study not only demonstrates the autochthonous existence of the zoonotic parasites *T. gondii* and *E. cuniculi* in voles in Vorarlberg, it also provides the first evidence of an occurrence of *N. caninum* in animals of the subfamily Arvicolinae, and it is an additional contribution to investigations of the sylvatic cycle of *N. caninum*.

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Introduction

The parasite fauna of rodents include several protozoan species of significant relevance for human and veterinary medicine, especially as a causative organism of zoonotic diseases such as toxoplasmosis or neosporosis. *Neospora caninum*, *Toxoplasma gondii*, and *Encephalitozoon cuniculi* display an affinity to the central nervous system and infect a wide range of domestic and wild mammals. Although the common vole (*Microtus arvalis*) is the most abundant mammalian species in Austria, basic knowledge about the protozoan parasite fauna of central European voles is limited. Common voles mainly inhabit grassland and agricultural fields, while water voles prefer open, treeless wetland habitats but are also found in agricultural areas. Several definitive hosts of the examined parasites act as predators of these voles. In several regions, common voles are known to be the most important diet ingredient (33–40%) of feral domestic cats (Biró et al. 2005). Farm cats as well as foxes are known to make water voles their favorite prey wherever vole population density is high (Weber and Dailly 1998). Nevertheless, reliable data about the role of voles in the epidemiology of the zoonotic diseases mentioned above are lacking.

T. gondii is an obligate, intracellular coccidian parasite which uses cats as its definitive host and a variety of mammals and birds as intermediate hosts. *T. gondii* is an important pathogen in humans, especially putting pregnant

women and AIDS patients at risk (Aspöck et al. 2002; Aspöck and Hassl 1990). One method of infecting humans is the ingestion of oocysts, which are temporarily shed via feces from infected cats. However, stray cats may also become infected by feeding on wild mammals or birds, especially on rodents. Several investigators have examined the distribution of *T. gondii* in wild and domestic animals in Austria (e.g., Werner et al. 1973; Frank 1978; Edelhofer 2004), but only once was a *T. gondii* infection of a common vole reported from eastern Austria (Frank 1978).

N. caninum, a coccidian species closely related to *T. gondii*, has been known as a parasite of mammals since 1988. *N. caninum* is recognized as a pathogen of dogs, even causing fatal neuromuscular diseases, and of cattle, where it is a major cause of abortion (Dubey 2003). Although humans produce antibodies against *N. caninum* via natural antigen contact, there is no confirmed evidence of a zoonotic infection (Dubey et al. 2007).

Recently the sylvatic cycle of *N. caninum* was elucidated; it includes wild canides and ruminants. Dogs and coyotes (*Canis latrans*) were demonstrated to excrete oocysts of *N. caninum* as definitive hosts, and antibodies to *N. caninum* were found in a variety of canides including foxes (Gondim 2006; Wapenaar et al. 2006). At present, however, there have only been a limited number of reports of *N. caninum* in rodents of the subfamily Murinae (Huang et al. 2004; Hughes et al. 2006; Jenkins et al. 2007; Ferroglio et al. 2007; Romano et al. 2009). There is a lack of knowledge about the role of voles in the parasite's cycle.

Microsporidiosis has been known as a disease of mammals since 1922, mostly of domestic and laboratory animals (Didier et al. 2000). Soon after the start of the AIDS pandemic in 1985, microsporidia were recognized as an opportunistic human parasite. Infections with *E. cuniculi* were diagnosed in several immunodeficient patients, especially in patients undergoing an organ transplant, in AIDS patients, or in persons with an idiopathic $CD4^+$ T-lymphocytopenia (Mathis et al. 2005). Although *E. cuniculi* is primary known as a pathogen of rabbits, it has also been reported as a parasite in murid rodents (reviewed in Mathis et al. 2005), but there is no information about the prevalence of *E. cuniculi* in rodents in Austria. The aim of this study was to prove the evidence of *T. gondii*, *N. caninum*, and *E. cuniculi* in the brains of wild common voles (*M. arvalis*) and water voles (*Arvicola terrestris*) in a peri-urban area in western Austria.

Material and methods

Between September and December, 2004, a total of 411 voles were captured as part of a pest control program in the peri-urban area of three cities (Dornbirn, Hohenems, and Lustenau; Vorarlberg) in the most western part of Austria. Out of the collected animals, 268 common voles (*M. arvalis*) and 86 water voles (*A. terrestris*) were included in this study. The vole's carcases were stored at −18°C and brought to the Medical University of Vienna for necropsy and further examinations. Data about the sex, body weight, and length to the base of the tail were gathered for each vole during dissection. To exclude mix-ups with field voles (*Microtus agrestis*), species determination of *M. arvalis* was classified on the basis of the second molars of the upper jaw. Brain samples were collected under clean laboratory conditions and stored in 70% ethanol at room temperature. The samples were homogenized with an automated homogenisator (Precellys®24, Peqlab, Austria), and the genomic DNA was extracted within 24 h using QIAamp mini kits (Qiagen GmbH & Co KG, Germany). The DNA was stored at −20°C until further procedures.

The *T. gondii*–PCRs were carried out using primers bound to highly repetitive regions of the B1 gene described by Burg et al. (1989). Primers NP6 and NP21 (Müller et al. 1996) were used for the detection of *N. caninum* DNA. For evaluation of *E. cuniculi*, the primers MSP-3 and MSP-4B were used, which enable a species determination within the microsporidal LSU rRNA gene (Katzwinkel-Wladarsch et al. 1996, 1997; Franzen and Müller 1999). All gene amplifications were performed with a MolTaq Mastermix (Molzym GmbH & Co. KG, Germany) using previously reported protocols for *T. gondii* and *E. cuniculi* (e.g., Murphy et al. 2007). The PCR method used for the detection of *N. caninum* DNA has been reported previously (Müller et al. 1996). The amplification products were analyzed by silver staining after flat-bed polyacrylamide electrophoresis (Amersham Pharmacia, Austria).

A statistical analysis was performed in order to demonstrate frequencies of parasite distribution in the populations and to find statistically significant differences between voles infested with parasites and those without parasites. Frequency distributions were calculated by differentiating region and species. Significance analyses were performed by applying chi-square tests (95% confidence interval (CI)) whenever the test conditions were met. Those groups were differentiated by species and weight, length, or by sex.

Results and discussion

Six voles tested positive for *T. gondii*, i.e., two of 264 *M. arvalis* and four of 86 *A. terrestris* (Table 1). Overall, six rodents demonstrated positive results for *N. caninum*, i.e., four of 264 *M. arvalis* and two of 86 *A. terrestris*. Furthermore, 22 Arvicolinae were found to be positive for *E. cuniculi*, i.e., 16 of 264 *M. arvalis* and six of 86 *A.*

Table 1 Number of positive samples (*n*), prevalence (percent) with 95% CI for the different parasite species found in water and common voles in Dornbirn, Lustenau, and Hohenems in Vorarlberg, Austria

Location	Species	Number	+	
			%	95% CI
Toxoplasma gondii				
Dornbirn	*Microtus arvalis*	2	0.90	0.2–3.2
	Arvicola terrestris	1	16.67	3.0–56.3
Hohenems	*Microtus arvalis*	0	0.00	0.0–9.6
	Arvicola terrestris	3	4.05	1.4–11.3
Lustenau	*Microtus arvalis*	0	0.00	0.0–29.9
	Arvicola terrestris	0	0.00	0.0–39.0
Neospora caninum				
Dornbirn	*Microtus arvalis*	4	1.79	0.7–4.5
	Arvicola terrestris	0	0.00	0.0–39.0
Hohenems	*Microtus arvalis*	0	0.00	0.0–9.6
	Arvicola terrestris	2	2.70	0.7–9.3
Lustenau	*Microtus arvalis*	0	0.00	0.0–29.9
	Arvicola terrestris	0	0.00	0.0–39.0
Encephalitozoon cuniculi				
Dornbirn	*Microtus arvalis*	10	4.48	2.5–8.1
	Arvicola terrestris	1	16.67	3.0–56.3
Hohenems	*Microtus arvalis*	5	13.89	6.1–28.7
	Arvicola terrestris	3	4.05	1.4–11.3
Lustenau	*Microtus arvalis*	1	11.11	2.0–43.5
	Arvicola terrestris	2	33.33	9.7–70.0
Total	*Microtus arvalis*	268		
	Arvicola terrestris	86		

Number of water voles (*A. terrestris*) examined: n=86 (n_{Dornbirn}=6; n_{Hohenems}=74; n_{Lustenau}=6). Number of common voles (*M. arvalis*) examined: n=268 (n_{Dornbirn}=223; n_{Hohenems}=36; n_{Lustenau}=9)

terrestris. One water vole captured in Hohenems tested positive for both *T. gondii* and *N. caninum*.

For calculating statistically significant differences in the groups being differentiated by species, weight, sex or length, the test conditions for the groups with *T. gondii* and *N. caninum* were not met due to the low prevalence of parasites in those hosts. A significant difference in the weight and length of voles with and without *E. cuniculi* was not observed. Furthermore, there was no significant difference of *E. cuniculi* infections and the vole's sex.

Although *T. gondii* is known to be a very common parasite in central Europe and between 33% and 40% of the human population in Austria is infected (Aspöck et al. 2002), the most important method of transmission (eating pseudocysts in raw or undercooked meat or oral ingestion of oocysts from cat feces) is not apparent. In Austria, there is no tradition of consumption of raw meat, and within two decades, the seroprevalence in pigs has decreased from 13.7% to <1% (Edelhofer 2004). This leads to the assumption that the main sources of human infections are oocysts excreted by cats. Hejlícek et al. (1997) reported a seroprevalence of 1% in *M. arvalis* in the Czech Republic, and in a recent study, Reperant et al. (2009) confirmed the detection of antibodies to *T. gondii* in 5% of water voles and 2.5% of common voles in the Canton of Geneva, Switzerland. In our study, we were able to detect comparable prevalence results of *T. gondii* in 0.7% of the common voles and 4.7% of the water voles, which strengthens the suggestion that the excretion of oocysts by stray cats might be the main source of infection. Cats are the main source of environmental contamination, and infection rates in cats indicate the infection rate with *T. gondii* in avian and rodent populations because cats become infected by eating those animals (Hill and Dubey 2002). Newer studies report that small wild rodents seem to be of high importance for the maintenance of the sylvatic cycle of *T. gondii* and serve as valuable indicators for the grade of environmental contamination with zoonotic parasites of carnivores (Reperant et al. 2009).

The most abundant wild canide species in Europe is the red fox (*Vulpes vulpes*). In some habitats, *Microtus* voles act as the main food source of red foxes (Lanszki 2005). Several prevalence studies have described infections with *N. caninum* in red foxes using serological techniques (e.g., Sobrino et al. 2008) and/or molecular biological methods (e.g., Murphy et al. 2007). A study on the seroprevalence in foxes and dogs in Austria revealed an infection rate of 3.6% in dogs, but out of 94 foxes examined, not a single one was serologically positive for this coccidian parasite (Wanha et al. 2005). Only a handful of studies reported *N. caninum* in wild murine rodents such as rats, house mice, and field mice. We found DNA of *N. caninum* in 1.5% of the common voles and 2.3% of the water voles, which is comparable to the results of Hughes et al. (2006) in mice and rats. Ferroglio et al. (2007) detected prevalences up to 13.8% of *N. caninum* in rats, mice, and field mice in western Italy. Overall, we were able to detect this pathogen for the first time in rodents in Austria. To the best of our knowledge, this is the first worldwide report of infections with *N. caninum* in *M. arvalis* and *A. terrestris* and in general in the subfamily Arvicolinae.

Although *E. cuniculi* is unlikely to be a common parasite in humans, its zoonotic role is clear (Mathis et al. 2005). It has been documented to infect a wide range of mammals and is regularly diagnosed in laboratory rodents, especially in hamsters, mice, and rats. There are only a few reports of *E. cuniculi* infections in wild rodents. Muller-Doblies et al. (2002) found *E. cuniculi* in one out of 30 wild rats in Switzerland, and one serological study in Iceland showed antibodies against this parasite in 4% of a population of *Apodemus sylvaticus* and in 9% of the house mice (*Mus musculus*) examined (Hersteinsson et al. 1993).

The results of our study can be summarized:

1. The occurrence of *T. gondii* infections in common and water voles were confirmed.
2. *N. caninum* was evaluated in rodents of the subfamily Arvicolinae (*M. arvalis* and *A. terrestris*). We are able to report *N. caninum* parasitoses in rodents in Austria for the first time ever.
3. For the first time, *E. cuniculi* was detected parasitizing rodents in Austria.

Thus, this study represents not only a contribution to the knowledge of the protozoan parasite fauna of Arvicolinae in Austria but it also documents the autochthonous distribution of parasites, which are of relevance for human and veterinary medicine.

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Ethical standards All voles were caught in 2004 in accordance with the Vorarlberger provincial law as published in Lg Bl. Nr. 50/2002.

Conflict of interest The authors declare that they have no conflict of interest.

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