Common voles (*Microtus arvalis*): Important reservoir for *Tula orthohantavirus* and other zoonotic pathogens

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CONTENT

LIST OF ABBREVIATIONS

ANDV	Andes orthohantavirus
BP	Before Present
C	Central lineage
CDC	Centers for Disease Control and Prevention
CEN.N	Central North
CEN.S	Central South
CI	Confidence Interval
DOBV	Dobrava-Belgrade orthohantavirus
CPXV	Cowpox virus
E	Eastern lineage
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
EST.N	Eastern North
EST.S	Eastern South
FUSV	Fusong orthohantavirus
H(C)PS	hantavirus (cardio)pulmonary syndrome
HFRS	hemorrhagic fever with renal syndrome
HTNV	Hantaan orthohantavirus
ICTV	International Committee on Taxonomy of Viruses
IFA	Immunofluorescence assay
IFN	Interferon
IgG	Immunoglobulin G
KHAV	Khabarovsk orthohantavirus
LCMV	*Lymphocytic choriomeningitis* mammarenavirus
ORF	open reading frame
PHV	Prospect Hill orthohantavirus
p.i.	Post infection
PUUV	Puumala orthohantavirus
RIG-I	retinoic acid inducible gene-I
RKI	Robert Koch-Institute
RNA	ribonucleic acid
RT-PCR	reverse transcription - polymerase chain reaction
SEOV	Seoul orthohantavirus
SNV	Sin Nombre orthohantavirus
STEC	Shigatoxin producing Escherichia coli
SUMO-1	Small ubiquitin-related modifier 1
TATV	Tatenale orthohantavirus
TBK1	TRAF-family member-associated NFκB activator binding kinase 1
TNF	tumor necrosis factor
TRAF3	TNF receptor associated factor 3
TULV	Tula orthohantavirus
Ubc9	Ubiquitin conjugating enzyme 9
VTEC	Verotoxin producing Escherichia coli
WHO	World Health Organisation

1. INTRODUCTION

1.1. Zoonoses

According to the World Health Organisation (WHO, 2020), about 14 % of all global deaths in 2019 were caused by infectious diseases. Zoonoses- diseases that are transmitted from animals to humans- are an important factor in these. It was estimated that about 61 % of 1415 species (viruses, bacteria, parasites) with known pathogenicity to humans with diverse routes of transmission are zoonotic (Taylor et al., 2001). The majority of zoonotic pathogens are transmitted indirectly (e.g., inhalation of particle of contaminated soil or ingestion of contaminated water), 35 % can be transmitted by direct contact to infected animals, 22 % are transmitted by vectors and for 6 % the transmission route is currently unknown (Taylor et al., 2001). In the future, surveillance and prevention of zoonotic diseases may be of even higher importance than now. Anthropogenic influence is changing landscapes, species distribution and composition of animal communities and is an important factor contributing to the emergence of infectious diseases (Thompson, 2013).

Of the emerging pathogen species 75 % are considered zoonotic (Taylor et al., 2001). Especially rodents may play an important role in future disease prevention strategies. Including more than 2279 species in 489 genera- almost half of all living mammal species- the order Rodentia is the most successful group, with members all over the world on all continents except Antarctica (Wilson and Reeder, 2005). At least 68 zoonotic viruses are associated with specific rodent host (Luis et al., 2013), some of them (such as hantaviruses causing the hantaviral (cardio)pulmonary syndrome, H(C)PS) with high fatality rates. Rodents are also the reservoir for many zoonotic bacteria (for example *Leptospira* spp., *Borrelia* spp., *Rickettsia* spp., *Anaplasma phagocytophilium*, *Salmonella* spp., *Campylobacter* spp., *Coxiella burnetii*, *Escherichia coli* (STEC/VTEC), *Francisella tularensis*, *Yersinia pestis*) as well as (endo)parasites (for example *Toxoplasma gondii*, *Babesia* spp., *Cryptosporidium* spp., *Trypanosoma cruzi*, *Leishmania* spp., *Giardia lamblia*, as well as different tapeworms, nematodes and trematodes) (Meerburg et al., 2009a; Recht et al., 2020; Helmy et al., 2018).

Close contact to humans and the progressing globalization might intensify conflicts between rodents and humans, when humans extend their civilization further into rodent habitats and refuges. Apart from their economic significance due to harvest losses or structural damages caused by their gnawing activities (Singleton et al. 2003; Meerburg et al., 2009b), pathogens transmitted from rodents to livestock may harm the animals as well as their handlers. While infection with *C. burnetti* in adult livestock is usually mild to asymptomatic, it might cause pneumonia, abortion, stillbirth or birth of weakened offspring (Arricau-Bouvery and Rodolakis, 2005). Infected livestock, in turn, is an important source for human Q fever disease outbreaks (Schimmer et al., 2010; Hellenbrand et al., 2001). Rodents are also frequently associated with outbreaks of leptospirosis in humans not only in developing countries but increasingly so in developed countries (Desai et al., 2009; Katelaris et al., 2020; Dupouey et al., 2014; Nau et al., 2019). While they do not transmit the bacteria directly, they contaminate soil or water bodies humans have contact with or ingest (Ullmann and Langoni, 2011; Haake and Levett, 2015). Inhalation of aerosols contaminated with rodent excreta as well as direct contact to rodents (wild-living as well as pets) transmits another important emerging viral pathogen to humans: *Lymphocytic choriomeningitis mammarenavirus* (LCMV), which is one of the most common causes of aseptic meningitis in humans and a danger especially during pregnancy as it can cause spontaneous abortion, fetal deformities and lasting neurologic sequelae of newborns (Vilibic-Cavlek et al., 2021). Persistently infected rodents can shed high quantities of LCMV during their lifetime (Lapošová et al., 2013).

Rodents are also important reservoirs for emerging diseases, they do not transmit directly or indirectly. While bartonellosis can be transmitted directly from animal to humans in some cases (for example cat scratch disease), most cases are arthropod-borne. Several different *Bartonella* spp. species have been identified in various rodent species and it has been reported that rodent reservoirs can be reinfected with a different *Bartonella* spp. species shortly after clearing the previous infection (Saisongkorh et al., 2009; Birtles et al., 2001). Lyme borreliosis, transmitted by ticks is a major public health concern in Europe- some countries report incidences of up to 155 cases

per 100,000 inhabitants per year (Stanek and Strle, 2003). Several *Borrelia* spp. species have been associated with human disease and have been detected in different rodent species acting as reservoir (Higgins, 2004). Another flea- or tick-borne pathogen is gaining importance in Europe: *Rickettsia* spp. While those obligate intracellular living bacteria might be beneficial to their arthropod host, several *Rickettsia* spp. species are pathogenic to human. There are open questions to answer when addressing surveillance and prevention strategies. The exact pathogenesis for example remains elusive (Legendre and Macaluso et al., 2017) or how many pathogenic *Rickettsia* spp. species exist is still unclear- pathogenic potential for some could only be clarified 60 years after their initial discovery (Paddock et al., 2004) and research is still describing new species in new hosts and in regions where they could not be detected before (Blanco and Oteo, 2006; Brouqui et al., 2007; Oteo and Portillo, 2012). Since *Rickettsia* spp. are found in several rodent species, some of the commensal species living in close contact to humans, they might play an important role as wildlife reservoir (Azad and Beard et al., 1998; Schex et al., 2011; Svoboda et al., 2013; Milagres et al., 2013; Kuo et al., 2015)

Introduction of generalist species to other parts of the world might also bring pathogens to new places. *Seoul orthohantavirus* (SEOV), a hantavirus associated with Norway rats (*Rattus norvegicus*), was first described in Asia, by now it has been reported in rat populations in Europe, Africa as well as both American continents (e.g., Childs et al., 1989; Costa et al., 2014; Dupiney et al., 2014; Heyman et al., 2009; Jameson et al., 2013; Diagne et al., 2020; Lundkvist et al., 2013). Additionally, several cases of infection with poxviruses (e.g., monkey pox virus or cowpox virus (CPXV)) caused by companion animals has been described in different countries in the last years (Essbauer et al., 2010; Tack and Reynolds, 2011; Campe et al., 2009; Ninove et al., 2009).

1.2. Hantaviruses

1.2.1. Rodent reservoir host

Hantaviruses (family *Hantaviridae*) are enveloped viruses with single-stranded RNA genome that consists of three segments that are usually associated with a single host species. Hantaviruses can be

transmitted directly between rodents via biting. Scars have been found to be associated with infection status of the respective rodent individual, especially for males (Khalil et al., 2014; Bennett et al., 1999; Papa et al., 2000; Hinson et al., 2004). But indirect transmission via the environment also plays an important role (Kallio et al., 2006b; Forbes et al., 2018). Studies have shown that infected animals are usually closer related to each other than the uninfected cohort. Thus, social behavior like grooming, burrow sharing and huddling together during the cold season might contribute to the spread of infection (Root et al., 2004; Deter et al., 2008; Yanagihara et al., 1985).

Viral RNA can be detected in urine, feces, and saliva of experimentally as well as naturally infected animals (Meyer and Schmaljohn, 2000; Hardestam et al., 2008). Upon a fresh infection there is usually a short viremic phase of about two weeks where virus can be detected in blood (Lee et al., 1981; Meyer and Schmaljohn, 2000; Schmaljohn, 1988; Forbes et al., 2018). Persistence is probably established during the first three or four weeks of infection. Viral RNA was detected up to one year in kidney, lung and saliva, but the shedding is not always continuously, depending on the hantavirus species (Meyer and Schmaljohn, 2000).

Virus prevalence in overwintered animals is often higher than in other seasons (Olsson et al., 2002; Chassnovnikarova et al., 2013; Kallio et al., 2010). Winter mortality, a reduced immunocompetence during that season, closer social interactions in shared burrows and recruitment of uninfected young to the population size during breeding season might result in that higher anti-hantavirus antibody and/or RNA prevalences in spring (Lehmer et al., 2010; Razzauti et al., 2013; Madhav et al., 2007). Infected dams transfer antibodies to their offspring either *in utero* and/or while nursing, with titres peaking two weeks after birth. Protection against hantavirus infection lasts at least eight to ten weeks after birth, an extended time span up to 3.5 months was reported for *Puumala orthohantavirus* (PUUV) (Zhang et al., 1988; Dohmae et al., 1993; Dohmae and Nishimune, 1995; Bernshtein et al., 1999; Kuenzi et al., 2005). Since the probability of infection is negatively correlated

to the presence of maternal antibodies, they might play an important role in prevalence fluctuation in naturally infected populations (Kallio et al., 2010).

It is widely accepted that hantaviruses persistently infect their host without causing any obvious symptoms, but several studies contradict that assumption. There are reports about reduced survival rates of bank voles (*Myodes glareolus*, Syn.: *Clethrionomys glareolus*) infected with PUUV and deer mice (*Peromyscus maniculatus*) infected with *Sin Nombre orthohantavirus* (SNV) (Kallio et al., 2007; Tersago et al., 2011a; Douglass et al., 2001; Luis et al., 2012), lung edema and periportal hepatitis (Lyubsky et al., 1996; Netski et al., 1999) as well as growth retardation (Childs et al., 1989; Kanerva et al., 1998). Uninfected animals or animals protected by maternal antibodies reached sexual maturity faster while infected breeding females are in poorer condition and are less likely to reproduce with age. Thus, hantavirus infection could impair breeding success (Kallio et al, 2006a; Kallio et al., 2015; Dearing et al., 2009).

The transmission of a pathogen from its original host to another species may result in serious consequences and a disease outbreak. It is therefore important to investigate the host range of pathogens. An adaption to the new organism might drive pathogen evolution and create new variants with novel characteristics. Host switch events have been suggested to have happened in hantavirus evolution at multiple time points (Kang et al., 2009a, 2009b, 2011; Liu et al., 2012) and spillover infections are the basic prerequisite for adaption to a new host. There are various reports of spillover infections of European hantaviruses species from their respective host to other rodent species evidenced through RNA detection (e.g., Schmidt-Chanasit et al., 2010; Plyusnin et al., 1994; Schlegel et al., 2009, 2012a and 2012b; Song et al., 2002; Weidmann et al., 2005; Christova et al., 2015). Additionally, antibodies have been detected in a wide variety of non-host mice and vole species (e.g. Aberle et al., 1999; Childs et al., 1994; Jay et al., 1997; Klingström et al., 2002; Kuenzi et al., 1999; Niklasson et al., 1995), muskrats (Vahlenkamp et al., 1998), elk (Ahlm et al., 2000),

foxes (Escutenaire et al., 2000a), domestic cats and dogs (Leighton et al., 2001; Malecki et al., 1998), rabbits (Childs et al., 1994) and non-human primates (Mertens et al., 2011a).

Animal experiments have shown that the transmission of PUUV from bank voles to related species (field voles, *Microtus agrestis*) and DOBV (genotypes Kurkino and Belgrade) from striped field mice (*Apodemus agrarius*) and yellow-necked field mice (*Apodemus flavicollis*) to laboratory mice works well. Other rodent species (e.g., the Mongolian gerbil (*Meriones unguiculatus*) and Syrian hamster (*Mesocricetus auratus*)) have been shown to be susceptible to hantavirus infection when experimentally inoculated, but the greater the phylogenetic distance between the rodent species the less likely is a natural infection (Klingström et al., 2002; Forbes et al., 2013; Sanada et al., 2011; Zhu et al., 1984).

1.2.2. Hantavirus disease in humans

To date, 53 species of hantaviruses (order Bunyavirales, family *Hantaviridae*) are accepted by the International Committee on Taxonomy of Viruses (ICTV) (Laenen et al., 2019). They are hosted by a wide variety of animal orders (from reptile to fishes to mammals) and on all continents except Antarctica. No human cases have been reported for Australia so far although antibodies reactive to hantaviral antigen could be detected in rodents as well humans. The respective virus species has not yet been isolated (Bi et al., 2005; Clement et al., 2019; Ulrich et al., 2002; LeDuc 1986).

Humans are usually a dead-end host for the virus. Mostly transmitted via aerosol inhalation of virus contaminated rodent urine or feces (Vaheri et al., 2013b) an infection may also occur after a rodent bite, but case descriptions are rare (Douron et al., 1984). The only exception to this rule is the *Andes orthohantavirus* (ANDV), where occasional human-to-human transmissions and even super-spreading events were reported (Padula et al., 1998; Martínez et al., 2020). While hantaviruses endemic to the Americas (often called “New World hantaviruses”) cause a severe illness (H(C)PS) with a mortality of 40 - 50 % (Wells et al., 1997; Nichols et al., 1993; Centers for Disease Control and

Prevention (CDC), 2012), European and Asian hantavirus infections ("Old World hantaviruses") causing hemorrhagic fever with renal syndrome (HFRS) are less fatal. Most European countries report human HFRS cases or at least hantavirus-specific antibodies in the human population (Vapalahti et al., 2003, Bi et al., 2008, Heyman et al., 2011). Two hantaviruses pathogenic to humans have been detected in European Murinae rodents: *Dobrava Belgrade othohantavirus* (DOBV), strain Dobrava in yellow-necked field mice and strain Kurkino in striped field mice, and SEOV in Norway rats (*Rattus norvegicus*). Case fatality rates range from less than 1 % for DOBV-Kurkino to 1-2 % for SEOV and up to 14 % for DOBV-Dobrava (Tkachenko et al., 2019, Song et al., 1984, Bi et al., 2008). Another two pathogenic hantaviruses could be detected in voles (family Cricetidae): PUUV in bank voles and *Tula orthohantavirus* (TULV) in common voles (*Microtus arvalis*). Most of the approximately 100,000 annual HFRS cases in Europe are caused by PUUV (Bi etal., 2008), the case fatality rate ranges between 0.1 – 0.4 %. TULV has not been associated with patient's deaths so far.

Especially for hantaviruses with low pathogenicity it is difficult to determine a realistic number of human infections. Following the German Infection Protection Act taking effect in 2001, hantavirus infections were a notifiable disease in Germany. Since then, 16702 confirmed human infections have been reported to the Robert Koch-Institute (RKI) (data status: 24.05.2022) but in a seroprevalence study of Zöller et al. (1995) nearly 2 % of the studied sera were shown to have antibodies against hantaviruses. An even higher prevalence was detected for risk groups (up to 9 - 9.5 %) (Zöller et al., 1995; Mertens et al., 2011b). Comparing these results to the reported cases and the population of Germany it seems likely that there are a lot of unreported cases because the symptoms are mild and unspecific and are therefore not correctly diagnosed. A study in Finland reporting that only 52 % percent of serological diagnosed PUUV patients are in need of hospital care is supporting the hypothesis of a mild, underdiagnosed disease (Mustonen et al., 2013).

Epidemic outbreaks with 2000 to 3000 cases have also been described to occur in several places with a certain cyclicity of 2-4 years (Hukic et al., 2010; Heyman et al., 2009, 2012; RKI: Surv-Stat@RKI 2.0, https://survstat.rki.de). Virus sequences isolated from patients are closely related to those of rodents captured near the assumed area of exposure and peak years of human cases have been described to coincide with high rodent population densities (Krüger et al., 2013; Olsson et al., 2010; Schilling et al., 2007; Tersago et al., 2011b; Reil et al., 2015), highlighting the importance of host population dynamics. In northern Europe these dynamics seem to be mainly driven by predators, while climate and food availability seem to be the key factor in temperate Europe (Lambin et al., 2006). Certain climatic condition favor high fruit production of beech and oak trees ("mast years"), thereby providing plenty of food for bank voles, the PUUV reservoir, and promoting best survival and early breeding conditions (Clement et al., 2009; Tersago et al., 2009).

Prevalence in the reservoir host is influenced by population density and habitat. Specific habitat factors may pose a greater infection risk for humans than others. Continuous forest environments, reduced biodiversity and young forests (25-30 years) are associated with the highest prevalence and PUUV infection rates in bank voles (Reusken and Heyman, 2013). Human and vole behaviour also needs to be considered when predicting exposure and infection risk. In Western, Central and Eastern Europe peaks of human infections are usually in summer (Kallio et al., 2009). This is in line with the finding that the volume of inhalable particles is greater in spring and summer than in autumn and winter season as well as in peridomestic environments compared to sylvatic environments. Simply moving around might be already an efficient way of transmission as walking is producing more particles than sweeping for example (Richardson et al., 2013). Greater human exposure during outdoor and recreational activities in summer months might also contribute to a higher infection rate during this season (Olsson et al. 2003). In contrast to temperate Europe, Fennoscandia is experiencing winter peaks, when wild rodents seek shelter in human housings (Olsson et al., 2010).

1.3. *Tula orthohantavirus* (TULV)

1.3.1. Host species: common vole

The common vole is one of the most abundant European mammals, distributed from sea level to about 2600 m throughout most of the European continent with exception of Mediterranean regions, Fennoscandia, and most of Great Britain, except the Orkney Islands where it has been introduced approximately 4,800±120 Before Present (BP) (Haynes et al., 2003; Mitchell-Jones et al., 1999; Spitzenberger et al., 2001).

Preferred habitats are open grassland, agricultural land, and short meadows where common voles live underground in shallow burrows and runway systems connecting different feeding areas. Their population densities are usually increasing from spring to autumn, and they are infamous for their cyclic super-abundant densities ("outbreaks") every 3-5 years. Peak densities of more than 2000 animals/ha have been reported, making the amplitude of population fluctuation the highest of all vole species by far (Jacob and Tkadlec, 2010; Jacob et al., 2014). These explosive outbreaks, causing considerable economic losses in crop production (Jacob and Tkadlec, 2010), are possible because females reach sexual maturity at the early age of two weeks and because of the highly flexible social behaviour of these animals. In times of high densities, the high territoriality of both sexes is reduced, and they form large groups and colonies of related individuals (Tkadlec and Zejda, 1995; Frank, 1957). The population dynamics are mainly influenced by weather conditions that have direct or indirect influence on the survival (e.g., floodings of burrows after heavy rainfall, plant growth as cover and food) and habitat parameters and its ability to support the needs of voles as well as generalist and specialist predators such as foxes, kestrels, and least weasels (Kidawa and Kowalczyk, 2011; Korpimäki, 1985; Delattre et al., 1999; Ylönen et al., 2019).

Present in Europe for at least 500,000 years (Kowalski et al., 2001) and constricted to different refuges during the last glacial period, diversification of this species led to distinct evolutionary lineages of this species with different divergence times. Eastern, Central, Italian, and Western lineage

are strongly supported by autosomal as well as mitochondrial DNA markers. The Western lineage is the oldest, the split between Central and Eastern lineage the most recent (Fink et al., 2004; Heckel et al., 2005; Lischer et al., 2014). These subpopulations are not isolated; there are contact zones between different lineages although at least partial reproductive isolation of different lineages was reported with exception of the least divergent Central and Eastern lineages (Beysard and Heckel, 2014; Sutter et al., 2013: Saxenhofer et al., 2019 and 2022).

Usually, hantaviruses are associated with only one rodent host and so called “spillover” infections are rare events. But TULV RNA has been detected in 5 different Arvicolinae species apart from its main host, the common vole: Sibling voles (*Microtus levis* formerly *rossiaemeridionalis*), narrow-headed voles (*M. gregalis*), European pine voles (*M. subterraneus*), field voles, and water voles (*Arvicola amphibius*) (Plyusnin et al., 1994; GenBank Accession number AF442621; Song et al., 2002; Korva et al. 2009; Schmidt-Chanasit et al., 2010; Schlegel et al., 2012a). TULV has been reported to occur in field voles even without the presence of (infected) host animals at the studied site, which raises the question of a potential second host species for this virus (Schmidt-Chanasit et al., 2010).

1.3.2. *Tula orthohantavirus* - a neglected pathogen?

Several *Microtus*-associated hantavirus species have been described and acknowledged by the ICTV: *Prospect Hill orthohantavirus* (PHV) in meadow voles (*Microtus pennsylvanicus*), *Tatenale orthohantavirus* (TATV) in field voles, *Khabarovsk orthohantavirus* (KHAV) in the Maximowicz's vole (*Microtus maximowiczii*) and *Fusong orthohantavirus* (FUSV) in the reed vole (*Microtus fortis*). None of them could be linked to human disease so far, although non-human primates showed sign of mild, transient nephropathy after experimental inoculation with PHV (Yanagihara et al., 1988).

On the other hand, several studies on TULV proteins and their interaction with host cells as well as immune response elicited, are placing this virus somewhere between non-pathogenic viruses like PHV and pathogenic like HTNV and ANDV:

(I) Although the cytoplasmatic tail of the Gn protein is missing the degrons typical for pathogenic hantaviruses, it shows more similarities to Gn proteins of pathogenic than to non-pathogenic hantaviruses. Its inability to bind tumor necrosis factor (TNF) receptor associated factor 3 (TRAF3) is not interfering with its ability to interfere with the retinoic acid inducible gene-I (RIG-I) and the TRAF-family member-associated NFκB activator binding kinase 1 (TBK1)-directed early interferon (IFN) activation, indicating a new mechanism, different from the one used by pathogenic hantaviruses. Consequently, TULV can replicate successfully in human endothelial cells to similar levels (Matthys et al., 2011; Matthys and Mackow 2012).

(II) TULV is also interacting with SUMO-1 and Ubc9 like PUUV which might delay apoptosis (Kaukinen et al., 2003), although there are reports of TULV-induced apoptosis starting shortly after infection (day 1-7 post-infection (p.i.)) by triggering pro-apoptotic signals of endoplasmic reticulum (ER)-stress and involving the TNF-receptor-1-mediated signal pathway and caspase-8 activity (Li et al., 2004, 2005).

(III) The S segment has a second overlapping open reading frame (ORF) (+1) coding for a small non-structural (NSs) protein (Spiropoulou et al., 1994; Jääskeläinen et al., 2007; Vera-Otarola et al., 2012). It may have an important role in IFN response interference and thus, prolonging virus survival throughout more consecutive passages (Jääskeläinen et al., 2007, 2008). Further experiments hint that the NSs protein might also interact with various cellular factors involved in signaling and transport (Rönnberg et al., 2012). TULV NSs protein has been shown to suppress INF β promoter activity even more strongly than the PUUV NSs protein (Binder et al., 2021). Still, in direct comparison only PUUV NSs protein was able to significantly delay IFN β expression and activation of IFN-stimulated genes (Gallo et al., 2021).

(IV) On the other hand and in contrast to pathogenic hantaviruses, the cell motility is not influenced since TULV is using β_1 instead of β_3 integrins like non-pathogenic hantaviruses. Consequently, there is also no recruitment of inactive platelets by β_3 integrin bound virions (Gavrilovskaya et al., 2010).

(V) There is also no virus-mediated increased sensitivity of endothelial cells to the Vascular Endothelial Growth Factor (VEGF) and therefore no increased vascular permeability (Gavrilovskaya et al., 2008).

TULV can cause human infections under certain conditions. TULV-specific antibodies were detected in a healthy blood donor from a region in the Czech Republic, where TULV circulates (Vapalahti et al., 1996), in forestry workers, an occupational risk group for hantavirus infections, in Brandenburg as well as in a HFRS patient from the same federal state (Klempa et al., 2003; Mertens et al., 2011b) and a 12-year-old patient with exanthema and fever from Switzerland (Schultze et al., 2002). TULV RNA could be successfully detected from the blood of an immunocompromised HFRS patient in the Czech Republic (Zelena et al., 2013) as well as an immune-competent person in northern Germany (Hofmann et al., 2021).

2. OBJECTIVES

Common voles are a frequently occurring generalist species in landscapes shaped by agriculture in most parts of Europe and several territories of Asia. Common vole population density changes in 3–5-year cycles in temperate Europe. Those animals can reach extremely high abundances, making them an economically important species as they can cause significant agricultural damage and loss of harvest.

Among other pathogens, hantaviruses are an emerging zoonotic threat in Europe and common voles are the reservoir host of TULV, an orthohantavirus that differs from other hantaviruses in its ability to infect several different vole species instead of being closely associated with one specific host.

The aim of this study was to:

→ evaluate TULV distribution and prevalence in common vole populations in several European countries

→ clarify the host association and the potential of other vole species as equally suited host for TULV replication

→ investigate TULV prevalence dynamics in fluctuating host populations

→ evaluate the general capacity of common voles to house different zoonotic pathogens in comparison to other wild living rodent species

2. PUBLICATIONS

(I) HIGH GENETIC STRUCTURING OF TULA HANTAVIRUS

Schmidt S, Saxenhofer M, Drewes S, Schlegel M, Wanka KM, Frank R, Klimpel S, von Blanckenhagen F, Maaz D, Herden C, Freise J, Wolf R, Stubbe M, Borkenhagen P, Ansorge H, Eccard JA, Lang J, Jourdain E, Jacob J, Marianneau P, Heckel G, Ulrich RG

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ORIGINAL ARTICLE

High genetic structuring of Tula hantavirus

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Abstract Tula virus (TULV) is a vole-associated hantavirus with low or no pathogenicity to humans. In the present study, 686 common voles (*Microtus arvalis*), 249 field voles (*Microtus agrestis*) and 30 water voles (*Arvicola* spec.) were collected at 79 sites in Germany, Luxembourg and France and screened by RT-PCR and TULV-IgG ELISA. TULV-specific RNA and/or antibodies were detected at 43 of the sites, demonstrating a geographically widespread distribution of the virus in the studied area. The TULV prevalence in common voles (16.7 %) was higher than that in field voles (9.2 %) and water voles (10.0 %). Time series data at ten trapping sites showed evidence of a lasting presence of TULV RNA within common vole populations for up to 34 months, although usually at low prevalence. Phylogenetic analysis demonstrated a strong genetic structuring of TULV sequences according to geography and independent of the rodent species, confirming the common vole as the preferential host, with spillover infections to co-occurring field and water voles. TULV phylogenetic clades showed a general association with evolutionary lineages in the common vole as assessed by mitochondrial DNA sequences on a large geographical scale, but with local-scale discrepancies in the contact areas.

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Introduction

Hantaviruses (family *Bunyaviridae*, genus *Hantavirus*) were initially thought to be exclusively rodent-borne pathogens hosted by representatives of the families Muridae and Cricetidae [1]. The recent finding of novel hantaviruses in insectivores such as shrews and moles as well as bats raises questions about the origin and evolution of this group of viruses [2, 3].

Rodent-borne hantaviruses can cause two types of disease in humans, hantavirus cardiopulmonary syndrome (HCPS) and haemorrhagic fever with renal syndrome (HFRS). HCPS due to infection by New World hantaviruses, e.g., Sin Nombre virus (SNV) and Andes virus (ANDV), is associated with an average case fatality rate of about 40 % [4]. In Europe, hantaviruses are emerging pathogens with an increasing significance for human health [5], causing HFRS with differing case fatality rates, ranging from less than 1 % to 16 % [4, 6].

Hantaviruses associated with members of the vole genus *Microtus* have been detected in several parts of Europe, Asia and the North American continent. The North American California vole (*Microtus californicus*), meadow vole (*M. pennsylvanicus*) and prairie vole (*M. ochrogaster*) are known to harbour Isla Vista virus (ISLAV), Prospect Hill virus (PHV) and Bloodland Lake virus (BLLV), respectively [7]. In the Asian part of Russia and in China, three different hantaviruses have been detected in *Microtus* voles, with one species sometimes hosting more than one hantavirus: Khabarovsk virus (KHAV) was found in *M. fortis* and *M. maximowiczii*, Vladivostok virus (VLAV) in *M. fortis* and *M. oeconomus*, and Yuangjiang virus (YUJV) in *M. fortis* [8–12]. In Europe, two *Microtus*-associated hantaviruses have been described. Tula virus (TULV) was initially detected in common voles (*M. arvalis*) and sibling voles (*M. levis,* formerly *rossiaemeridionalis*) [13, 14]. A highly divergent TULV strain, designated as Adler virus, was recently discovered in common voles in Russia [15]. A second hantavirus, Tatenale virus, was found in field voles (*M. agrestis*) in England [16].

Like in all hantaviruses, the genome of TULV is partitioned into three segments: For TULV prototype strain Moravia, the small (S) segment, 1,831 nucleotides (nt) in length, codes for the nucleocapsid (N) protein, the medium-sized (M) segment of 3,694 nt codes for two glycoproteins (Gn und Gc), and the large (L) segment of 6,541 nt codes for the viral RNA-dependent RNA polymerase [17–19]. In addition, the S segment of all vole-derived TULV strains contains an overlapping open reading frame (ORF) coding for a putative nonstructural protein (NSs) that has been shown to enhance survival of the virus during passaging in interferon-competent cells [20, 21].

There is little knowledge about the pathogenicity of TULV for humans. TULV-specific antibodies have been detected in healthy blood donors in the Czech Republic [19] and in German forestry workers, a potential risk group for hantavirus infections [22]. Furthermore, one HFRS patient from Germany had TULV-specific neutralizing antibodies [23]. In addition, TULV RNA was detected in EDTA blood of an acutely infected, immunocompromised patient in the Czech Republic [24].

Corresponding to the wide distribution range of its main host, the common vole, TULV-specific nucleic acid has been detected in several European countries, but these studies usually included only one or a few trapping sites in a specific region (see Ref. [14] and [25–30] and references therein). Whereas most hantaviruses are host-specific and natural spillover infections are only rarely reported, TULV has been molecularly detected in a wide variety of other Arvicolinae species: *M. levis, M. gregalis*, *M. subterraneus*, *M. agrestis*, *Lagurus lagurus,* and *Arvicola* spec. [13, 31–35]. Aside from common voles, the most TULV infections have been reported in field voles (*M. agrestis*) in some places even without the presence of the main host species or with a larger number of infected field voles compared to sympatric common voles. It was therefore speculated that rather than being spillover infected, field voles might represent another host species that can enable successful TULV replication [29, 34].

The evolution of hantaviruses in relation to their rodent hosts is controversial. Often, the divergence of hantaviruses and their rodent hosts is interpreted as a consequence of co-evolutionary processes. Alternatively, host switching and subsequent adaptation processes are thought to be a reason for the observed divergence patterns [2, 7, 34, 36–38]. Previous investigations were mainly focused only at the species level of the reservoir, but co-evolutionary processes may also occur at the level of evolutionary divergence within species [7, 29, 34, 35, 37]. In Central and Eastern Europe, four main evolutionary lineages of common voles have been identified with both autosomal and mitochondrial DNA markers: the Western, Central, Italian and Eastern lineages [39–41]. So far, nothing is known about potential co-divergence between TULV and evolutionary lineages of the common vole.

Here, we describe large-scale serological and RT-PCR TULV screening of three potential reservoir species from

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Central and Western Europe, as well as phylogenetic analysis of TULV sequences and mitochondrial DNA sequences of common voles.

Materials and methods

Rodent trapping and dissection

Rodents were collected between 2004 and 2013 at 71 sites in 13 federal states of Germany, two sites in Luxembourg, and six sites in two administrative districts in France (Alsace and Midi-Pyrénées) in woodland and open field habitats (Fig. 1). Trapping of some of the animals has been described previously [42–45]. Rodent dissection was done according to standard protocols and resulted in the collection of heart, lung, liver, spleen, kidney, and brain, as well as tissue samples from the ear pinna and tail. Chest cavity fluid (CCF) was obtained by adding 1 ml of PBS [46]. For TULV-positive animals, TULV-negative animals from selected sites, and individuals with inconclusive morphological species identification, mitochondrial *cytochrome b* (*cyt b*) sequences were determined [47]. For samples that did not allow a morphological identification, a sex-determination PCR was performed following established protocols [48, 49].

Nucleic acid isolation

RNA extraction was performed using a modified QIAzol protocol. Briefly, RNA extraction was performed using 1 ml of QIAzol® Lysis Reagent (QIAGEN, Hilden, Germany) and sterilized steel beads of 0.5 cm diameter (Isometall, Pleidelsheim, Germany). After tissue homogenization, 200 µl of chloroform was added, and the sample was mixed and thereafter centrifuged for 15 min at 4 °C and 11,900 *g*. The resulting supernatant was mixed with 500 µl of cold isopropanol (-20 °C), incubated at -20 °C for 20 min, and centrifuged again at 4 °C for 10 min at 11,900 *g*. The resulting pellet was washed once with 1 ml

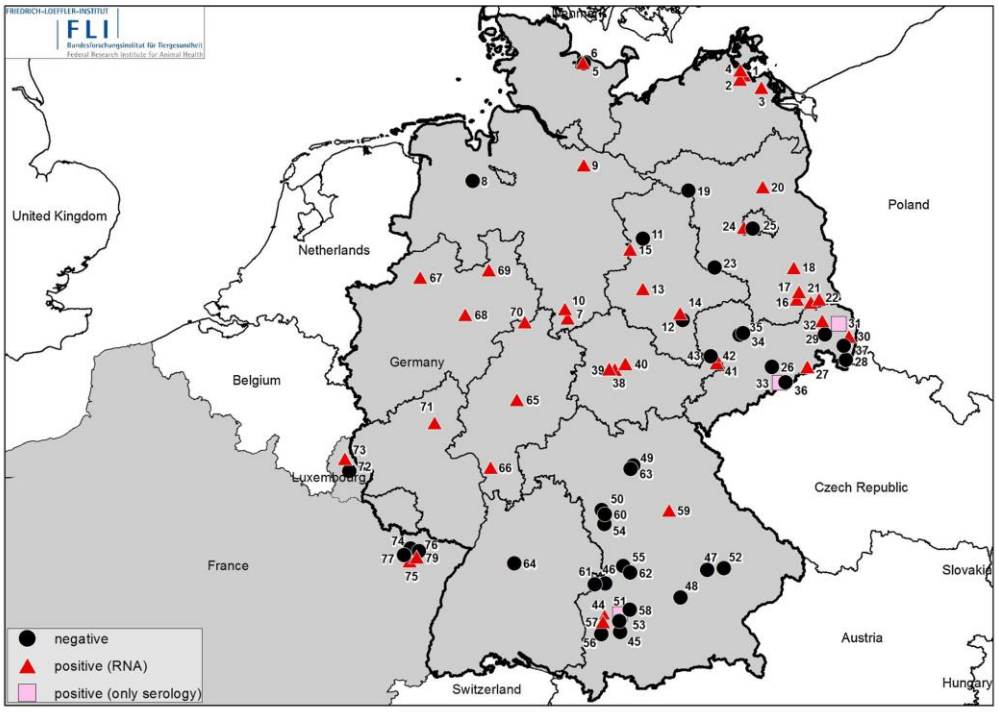


Fig. 1 Location of the main trapping sites in Germany, Luxembourg and France. An additional trapping site (#78) was located outside the range of this map in the Aveyron region in southern France

of 75 % ethanol and thereafter with 1 ml 99.8 % ethanol, dried at 56 °C for no more than 5 min, and resuspended in DEPC-treated water.

DNA for *cyt b* analysis was obtained from tissue samples using conventional chloroform DNA extraction or tail lysis overnight [47, 50]. Briefly, for extraction, all tissue samples were incubated overnight at 56 °C and 400 rpm in 300 µl of lysis buffer containing 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.45 % NP40, 0.45 % Tween 20 and 1 mg of proteinase K per ml.

RT-PCR and serology

Hantavirus RT-PCR assays of lung tissue samples were done according to previously described protocols for the PUUV/TULV S segment [51]. TULV-N-specific antibodies were detected in the CCF by IgG-ELISA using yeast-expressed, purified recombinant N protein of TULV strain Moravia [22, 34].

Statistical analysis

The significance of prevalence differences between sexes was investigated using the χ^2 test. Because of the low number of RNA-positive water voles, we used Fisher's exact test to evaluate the significance of prevalence differences between species.

Sequence determination and phylogenetic analysis

DNA sequencing was performed by the dideoxy-chain termination method using a BigDye Terminator v1.1 Kit (Applied Biosystems, Darmstadt, Germany) and Genetic Analyser 3130 and 3130xl sequencers (Applied Biosystems). When the direct sequencing approach failed, sequences were obtained after insertion of the RT-PCR product into the pCR®-TOPO®-vector and transformation of TOP10 cells according to the manufacturer's instructions (TOPO-TA-Cloning Kit, Invitrogen, Darmstadt, Germany). At least two plasmids per RT-PCR product were sequenced.

All generated data were subjected to a BLAST-mediated comparison of the novel sequences with sequences available in GenBank (http://www.ncbi.nlm.nih.gov). All TULV sequences were included in subsequent phylogenetic analysis, and identical sequences were excluded. For common vole lineage analysis, one to three representatives from each trapping site were chosen, with the exception of site #27, where mtDNA of 29 individuals was investigated. All TULV and mtDNA sequences were deposited in the GenBank database with accession numbers KU139527-KU139605 and KU139696-KU139816, respectively (Supplementary Tables 4 and 5).

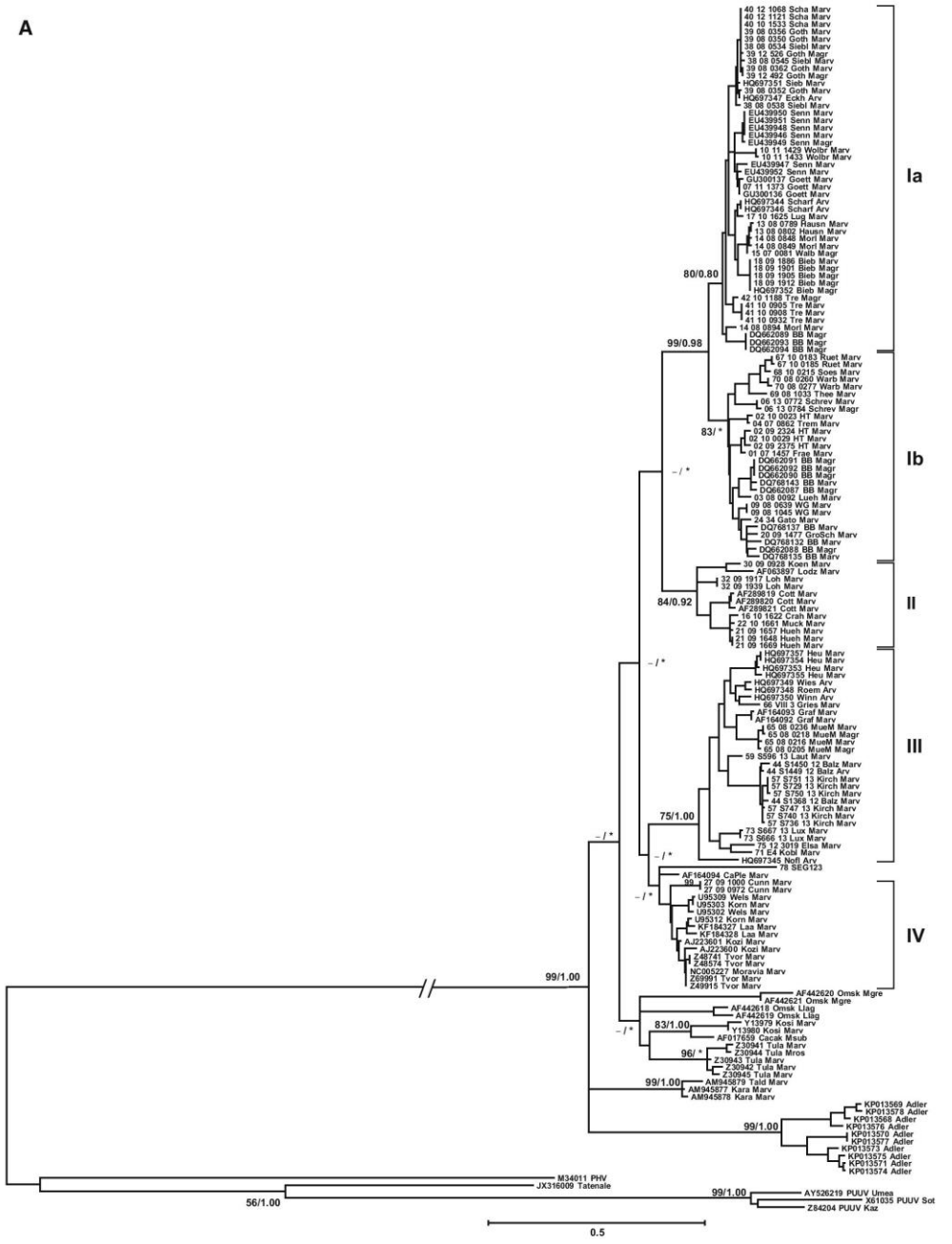
Nucleotide sequences were aligned using the ClustalW method implemented in BioEdit v7.2.5 [52] and revised manually. In addition to the novel sequences obtained in this study, representative sequences were obtained from GenBank, and these are labeled with accession numbers in Fig. 2 and Fig. 3. The final datasets used for analysis contained 158 S-segment sequences of 255-bp length for the TULV S-segment and 148 sequences of 763 bp from the *cyt b* gene of *M. arvalis*. Reference sequences for *cyt b* analysis were chosen as described in Ref. [34] and [53]. The outgroup sequences included Puumala virus (PUUV), Prospect Hill virus (PHV), and Tatenale virus for TULV and *M. gregalis* for the *cyt b* dataset. The best nucleotide substitution model to fit each data-set was determined with jModeltest v2.1.6 [54]. The Tamura and Nei model with a gamma-distribution model of among-site rate heterogeneity and a proportion of invariable sites (TrN+G+I) had highest scores for the TULV and *cyt b* data according to the Bayesian Information Criterion (BIC). MEGA 6 [55] was used for phylogenetic tree reconstruction based on maximum-likelihood (ML) algorithms with 1000 bootstrap replications. Phylogenetic relationships were also inferred using the Bayesian method implemented in MrBayes v3.2.2 [56] on the CIPRES platform [57], employing individual nucleotide substitution rate priors for each dataset. Four independent analyses were done for TULV and *cyt b* data, comprising each 10^7 generations of Markov chain Monte Carlo chains, sampled every 10^3 generations with a burn-in fraction of 25 %. For both datasets, the average standard deviation of split frequencies was lower than 0.01 in every run, and the potential scale reduction factor was in the range of 0.99-1.01 for all parameters, indicating that parameter convergence had occurred. Consensus trees were drawn with FigTree v1.4.2 [58].

Fig. 2 Maximum-likelihood (ML) phylogenetic tree based on TULV ▶ partial S-segment sequences (A) and association of TULV sequence clades with common vole evolutionary lineages (B). Novel sequences are labeled with sampling location, individual code, and host species. Published sequences obtained from GenBank are labeled with the accession number, location and host species. Bootstrap support for ML and posterior probabilities of Bayesian analyses are indicated for major branches only. — indicates bootstrap values <50; *indicates a different topology in Bayesian analysis. Clades Ia, Ib, II and III are major geographically coherent TULV clusters with adjacent distribution in Central Europe (see text). For GenBank accession numbers of the novel TULV sequences, see Supplementary Table 4

Results

Detection of TULV in common voles

To study the geographical distribution of TULV in its reservoir host, common voles were trapped in Germany, France and Luxembourg (Fig. 1, Table 1, Supplementary


A
Ia
Ib
II
III
IV
80/0.80
99/0.98
83/ *
84/0.92
75/1.00
99/1.00
83/1.00
96/ *
99/1.00
99/1.00
56/1.00
99/1.00
0.5

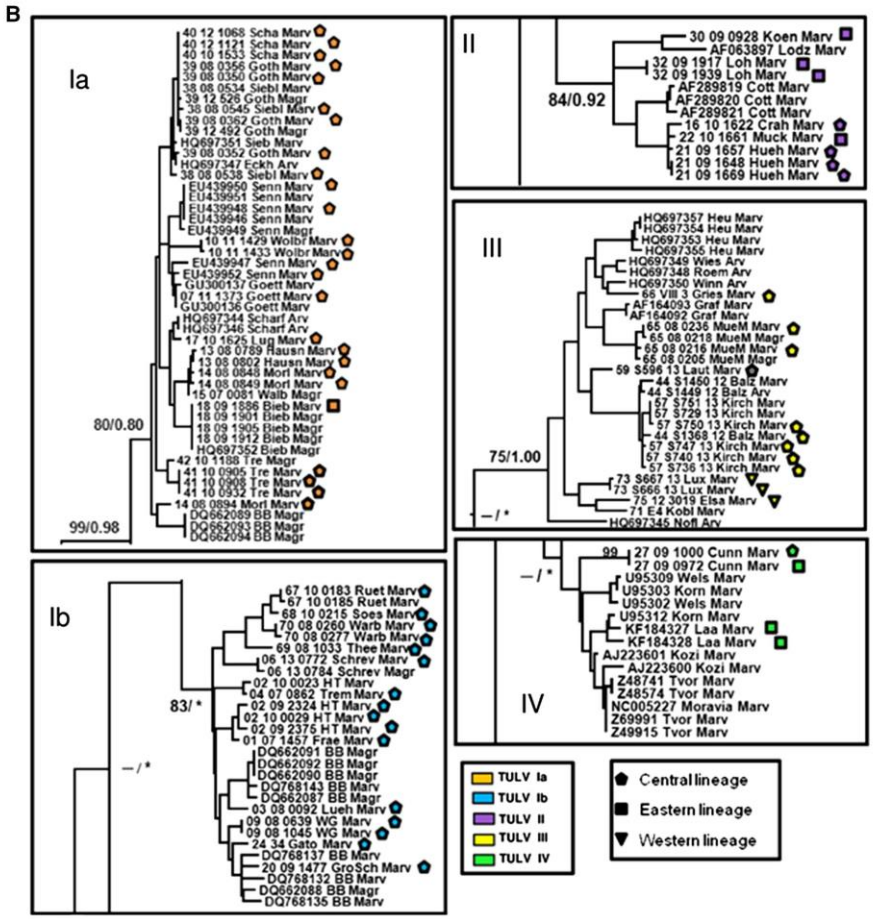


Fig. 2 continued

Table 1). The 686 common voles that were collected originated from 51 sites in 47 districts of 13 federal states in Germany (n = 654), six sites in two administrative regions of France (n = 26) and one site in Luxembourg (n = 6).

A total of 115 (16.7 %) common voles had signs of a previous or ongoing TULV infection (Table 1). Hantavirus-specific RNA and IgG antibodies were detected in 107 of 685 (15.6 %) and 49 of 670 (7.3 %) animals, respectively. Nine common voles (0.9 %) were positive in ELISA only without any detectable RNA. When focusing on the 15 locations where ten or more animals had been captured, the molecular prevalence ranged from 0 % to 37.5 % (mean: 14.2 %) and the seroprevalence ranged from 0 % to 19.0 % (mean: 5.3 %). TULV RNA could be detected in common voles from all three countries (Fig. 1, Supplementary Table 1). In contrast, TULV-specific antibodies were detected only in voles from Germany and Luxembourg. TULV-reactive antibodies were not found in any of the 26 animals from France, although TULV-RNA was detected in five animals originating from three trapping sites (Fig. 1, sites #75, #78, #79). For the RT-PCR-positive vole from site #79, no TULV sequence data were obtained after repeated attempts. Two out of six common voles from Luxembourg were RNA

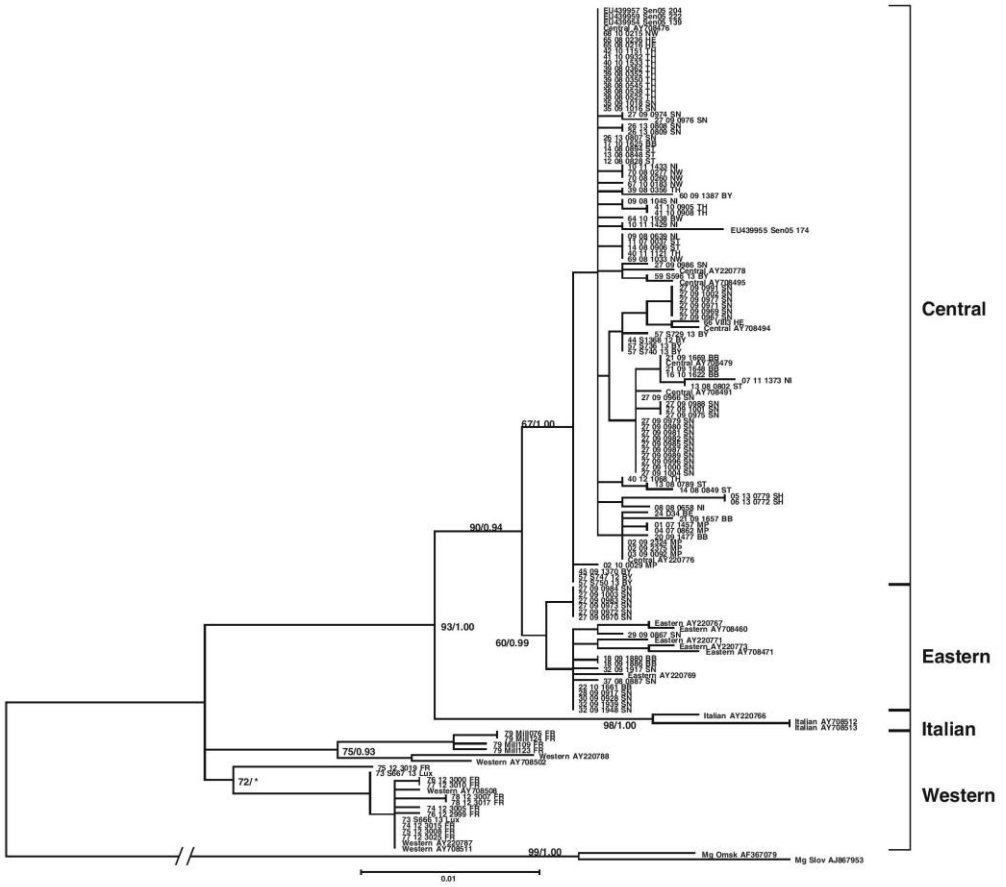


Fig. 3 Phylogenetic relationships of European *Microtus arvalis* based on *cytochrome b* sequences. The maximum-likelihood (ML) tree contains novel and published common vole sequences as references for the evolutionary lineages Central (C), Eastern (E), Italian (I) and Western (W), analogous to those reported in references 34 and 53. Labels for novel sequences start with the sampling location number, individual code and geographic region. Reference sequence labels specify the lineage and GenBank accession number. *M. gregalis* was used as an outgroup. Bootstrap support for ML and posterior probabilities of Bayesian analysis are indicated for major branches only. — indicates bootstrap values <50; *indicates a different topology in Bayesian analysis. For GenBank accession numbers of the *cytochrome b* sequences, see Supplementary Table 5

positive, one of them with parallel detection of TULV-reactive antibodies. In Germany, antibodies and/or TULV RNA could be detected in common voles at 34 of 51 trapping sites, with the majority of infected animals being antibody-negative (Supplementary Table 1). A total of 109 animals from Germany were positive for TULV-specific RNA and antibodies, and seven and 58 animals were exclusively antibody and RT-PCR positive, respectively. In addition, male common voles were more frequently found to be TULV positive than females (19 % vs. 14.7 %) (Table 2), but the difference was not significant ($\chi^2 = 2.319$, df = 1, $p = 0.128$).

Consecutive trappings of voles at one site in France (#75) and nine of 14 sites in Germany (#09, #10, #27, #39, #40, #41, #44, #57, #65) revealed a continuous presence or re-appearance of TULV infections (Supplementary Table 2). The presence of TULV RNA was monitored and detected in the common vole populations for one month (sites #10, #27, #44, #65), several months

Table 1 Results of serological and RT-PCR investigations for *Microtus arvalis* (Marv), *Microtus agrestis* (Magr) and *Arvicola* spec. (Arv) collected in Germany, Luxembourg and France

Species	No. of trapping sites	Total no. of voles analyzed	Sex		Weight range (g)		No. positive by serology only / total no. analyzed	No. positive by RT-PCR only /total no. analyzed	No. positive in both /total no. analyzed	Total no. positive by serology/ total no. analyzed	Total no. positive by RT-PCR /total no. analyzed	Total no. positive by serology and/ or RT-PCR
			Male	Female	Min	Max						
Marv	58	686	320	367	5	48	9/670	67/685	40/668	49/670	107/685	115/686
							1.3 %	9.8 %	6.0 %	7.3 %	15.6 %	16.8 %
Magr	37	249	134	115	16	47	10/246	4/249	9/246	19/249	13/249	23/249
							4.1 %	1.6 %	3.7 %	7.6 %	5.2 %	9.2 %
Arv	6	30	14	16	21	132	2/29	1/30	0/30	2/29	1/30	3/30
							6.9 %	3.3 %	0 %	6.9 %	3.3 %	10.0 %

[1] no sequence for one animal

(sites #9, #41, #57 and #75), and up to several years (sites #39, #40).

Detection of TULV in field voles

Field voles were successfully collected at 37 locations in Germany (n = 249). In total, 23 of 249 (9.2 %) field voles were found to have TULV-specific RNA and/or antibodies (Table 1). Thirteen field voles from four trapping sites in three German federal states were RT-PCR positive (5.2 %), and virus-specific antibodies were also present in nine of them. Ten additional animals from five federal states were positive by ELISA, but no viral RNA was detectable (Table 1, Supplementary Table 1). When focusing on the seven trapping sites with 10 or more field voles captured, the molecular prevalence ranged from 0 % to 14.0 % (mean 2.3 %), and the seroprevalence ranged from 0 % to 16.0 % (mean: 3 %). Similar to common voles, TULV infections were detected more often in males (11.2 %) than in females (7.0 %) (Table 3), but this difference was not significant ($\chi^2 = 1.326$, df = 1, $p = 0.25$).

At five of six trapping sites (#1, #18, #39, #44, #65) with TULV-positive field voles, TULV RNA was also detected in sympatrically occurring common voles. At the remaining site (#42) all four common voles were TULV negative. Inversely, at five of 11 sites with TULV-infected common voles (sites # 1, #18, #39, #44, #65), sympatric field voles showed signs of TULV infection. However, at five of the six sites without TULV-positive field voles, only 1-3 animals were found to be infected. At the remaining site (#41), all 21 field voles that were trapped were TULV negative.

TULV infection in water voles

Water voles were successfully trapped at four sites in Germany (n = 26) and both sites in Luxembourg (n = 4; Table 1). Two of the 29 water voles were positive in TULV-ELISA, but no RNA could be amplified from those animals (Table 1, Supplementary Table 1). A TULV sequence could be obtained from one additional animal, although there was no reaction in the ELISA. All three TULV-positive water voles originated from Bavaria, Germany, but the two seropositive animals and the RT-PCR-positive animal were captured at different trapping sites about 12 km apart (Fig. 1, sites #44 and #51).

At five of the six trapping sites, water voles occurred sympatrically with common voles (sites #26, #73), field voles (site #51) or both species (sites #44 and #65). At site #44, detection of TULV RNA in the single trapped water vole was accompanied by detection of TULV-specific RNA and antibodies in common voles and TULV-specific antibodies in field voles (Supplementary Table 1). At site

Table 2 Sex differences in the detection of TULV-specific RNA and antibodies in the vole species *Microtus arvalis* (Marv), *Microtus agrestis* (Magr) and *Arvicola* spec. (Arv)

	No. positive / total no. analyzed (percentage)					
	Male			Female		
	Serology	RT-PCR	Serology and/or RT-PCR	Serology	RT-PCR	Serology and/or RT-PCR
Marv	28/310 (9.0)	57/319 (17.9)	61/320 (19.1)	21/361 (5.8)	50/366 (13.7)	54/367 (14.7)
Magr	13/132 (9.8)	8/134 (6.0)	15/134 (11.2)	6/114 (5.3)	5/115 (4.3)	8/115 (7.0)
Arv	1/13 (7.7)	0/14	1/14 (7.1)	1/16 (6.3)	1/16 (6.3)	2/16 (12.5)

Table 3 Mean nucleotide (A) and amino acid (B) sequence identity of the S segment and nucleocapsid protein sequences, respectively, between the TULV clades in Central and Western Europe (for definition of the clades, see Fig. 2; for their geographical origin see Fig. 4)

	Ia	Ib	II	III	IV
A					
Ia	94.2 %	-	-	-	-
Ib	87.9 %	91.5 %	-	-	-
II	82.8 %	81.9 %	91.6 %	-	-
III	81.8 %	79.6 %	79.7 %	88.1 %	-
IV	80.2 %	80.7 %	82.2 %	83.3 %	92.0 %
B					
Ia	99.8 %	98.4 %	94.5 %	89.9 %	89.9 %
Ib	-	97.7 %	94.3 %	89.2 %	89.1 %
II	-	-	99.0 %	91.1 %	91.9 %
III	-	-	-	97.5 %	94.7 %
IV	-	-	-	-	98.2 %

#51, TULV-specific antibodies were detected in two of the 17 water voles, but not in the three field voles trapped there. At two sites (#65, #73), TULV infections were detected in common voles or common and field voles, but not in the few water voles collected sympatrically. At the remaining two sites (#26, #72) none of the water voles and common voles showed signs of a TULV infection.

Sequence comparison and phylogenetic analysis of TULV sequences

To analyze the phylogenetic relationships between TULV strains, S segment sequences of all RT-PCR-positive voles were compared to existing TULV sequences from Germany and other European countries. The phylogenetic tree revealed a strong geographic structuring of TULV sequences that was independent of the rodent species of origin (Fig. 2). Main cluster I represents the majority of TULV sequences originating from Germany and comprises two parapatric sister clades: Ia (central and eastern Germany) and Ib (northern, central and western Germany).

Main cluster II consists of novel sequences from the eastern part of Germany, published sequences from one site in this part of Germany [23], and prototype strain Lodz from Poland [59]. Cluster III contains sequences from southern Germany, Luxembourg, the Alsace region of France and a water-vole-derived TULV sequence from Switzerland [35]. TULV sequences from a trapping site in eastern Germany close to the Czech border (site #27), from Austria, and from the Czech Republic form cluster IV. The phylogenetic position of the novel sequence from southern France (site #78) could not be determined with confidence.

The intra-cluster proportion of variable sites ranged from 5.8 % to 11.9 % at the nt level and 0.2 % to 2.5 % at the amino acid level (Table 3). In contrast, the inter-cluster variability reached more than 20 % at the nt level and more than 10 % at the amino acid level, which exceeds the threshold level of 7 % established by the International Committee on Taxonomy of Viruses for hantavirus species definition based on the entire N protein sequence [60]. Sequence similarity at the nt and amino acid level was highest between the two subclades from Germany (Ia and Ib) and clade II from Eastern Germany and Poland (Tables 3A and 3B). The greatest differences were found between clusters Ia/Ib and III/IV, which differed on average by 8.9 % at the amino acid level and 20.3 % at the nt level (Tables 3A and 3B).

Co-segregation of TULV with evolutionary divergence in *M. arvalis*

To examine potential associations of TULV divergence with evolutionary divergence in the common vole, the *cyt b* sequences of selected animals were used for phylogenetic analysis together with reference sequences representing the evolutionary lineages Eastern, Central, Western and Italian (Fig. 3). The vast majority of *cyt b* sequences of common voles from Germany were identified as belonging to the Central lineage, and all investigated voles from France and

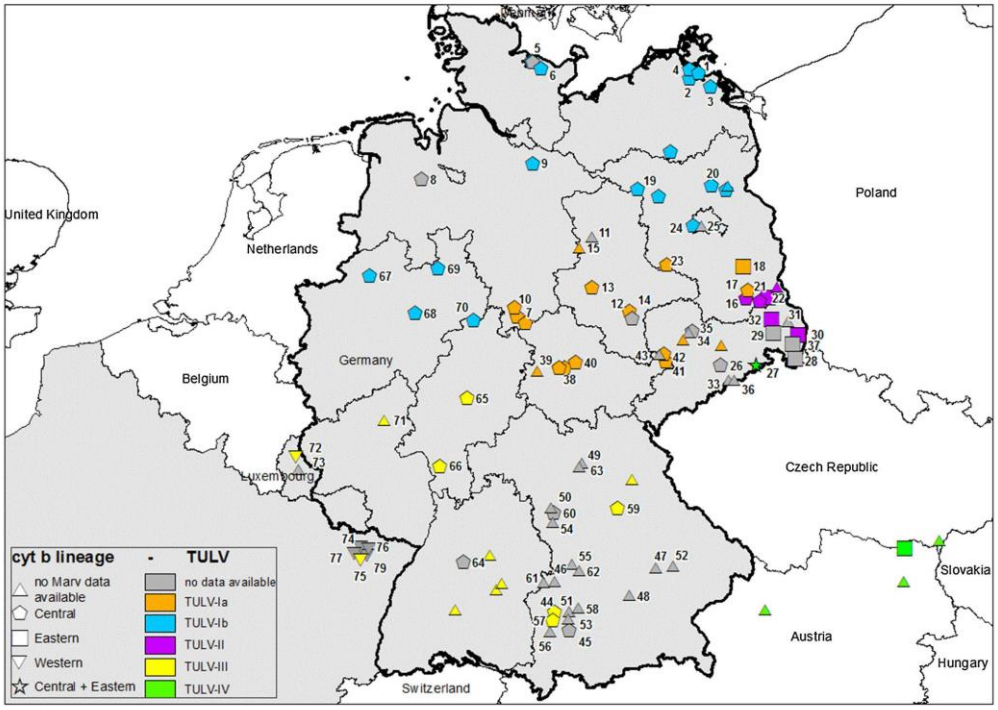


Fig. 4 Map showing the trapping sites of common voles infected with TULV of clusters Ia, Ib, II, III and IV and belonging to the evolutionary lineages Central, Eastern and Western

Luxembourg belonged to the Western lineage (Fig. 3 and Fig. 4). Interestingly, Central and Eastern evolutionary lineage *cyt b* sequences were identified in close proximity in the easternmost part of Germany, with sympatric occurrence of both lineages at site #27 (Fig. 4).

TULV clades showed a general association with certain evolutionary lineages of common voles. All animals harboring TULV of clusters Ia or Ib belonged to the Central lineage, except for one animal from Biebersdorf (site #18; Eastern lineage). TULV cluster III originated from common voles mainly of the Central lineage, with two from Luxembourg and one from France belonging to the Western lineage (sites #73 and #75). Sequences of cluster II are associated with the Eastern lineage but were also found in Central individuals trapped close to sites with Eastern lineage voles in western Brandenburg and southwestern Saxony (sites #28 and #79). TULV Cluster IV originated from Eastern-lineage hosts with one sequence from an animal of the Central lineage trapped at Cunnersdorf (site #27), Saxony. The TULV sequence from southern France originated from an animal of the Western lineage.

Discussion

Host specificity and spillover of TULV

In this study, we investigated three largely co-distributed vole species for the presence of TULV infections. The overall TULV prevalence for common voles (16.7 %) was higher than that for field voles (9.2 %) and water voles (10.0 %). In addition, the molecular prevalence for TULV differed significantly between species ($p < 0.001$; two-sided Fisher's exact test) and was again highest in common voles (15.6 %) compared to field voles (5.2 %) and water voles (3.3 %). Similarly, a previous study has shown a very low prevalence of TULV in water voles [35]. This may indicate a host preference of TULV for the common vole and the detection of TULV RNA in other vole species such as field and water voles being the result of spillover infections during the acute phase with active virus replication. In line with this assumption, almost all TULV-RNA-positive field voles were co-trapped with common voles or trapped at locations where TULV-RNA-positive

common voles were detected earlier and TULV sequences were relatively similar to those from common voles.

This observation of different prevalence in common voles versus field and water voles might also have been due to different interspecies interactions. Similar ecological characteristics and use of similar habitats can promote interspecific contact between common voles and other species directly (fighting) or indirectly (feces and urine) [61, 62]. Common voles and field voles might coexist in the same habitat, while encounters between water voles and other microtine species usually lead to a consistent dominance of the water vole and exclusion of permanent *Microtus* populations during the breeding season [63]. The water voles trapped in our study seemed to be sympatric with *Microtus* species at five out of six trapping sites, but these two species (water voles and *Microtus* spp.) had not shared the same habitat for an extended period of time (Supplementary Table 2). This might explain why we could detect TULV RNA in water voles at only one location.

A host function of field voles was discussed in previous studies, as TULV RNA was detected at higher prevalence in field voles than in common voles or in multiple field vole individuals in the absence of common voles [29, 34]. In this study, the molecular prevalence in field voles was in general much lower than in common voles. Furthermore, 53 % of field voles with TULV-reactive IgG antibodies were TULV-RNA negative, which is indicative of an earlier infection with virus clearance, but TULV-RNA could be detected in 81.6 % of TULV-IgG-positive common voles, which is typical of persistent infection in the reservoir. Furthermore, almost all field voles from sites without sympatrically occurring common voles or with only TULV-negative common voles were TULV negative.

The overall results of this study support the notion that common voles act as the preferential host for TULV. Collectively, these data do not support the idea that field voles serve as reservoir hosts with an equal ability to support TULV replication and persistence. Additional studies are needed to determine whether field voles may temporally function as a reservoir host with low viral load.

TULV in common vole populations

TULV was detected at 39 of 62 (62.9 %) sites with common voles, but with rather low prevalence compared to other vole-associated hantaviruses. While our results settle well within the reported range of TULV RNA prevalence of 8 %-37 % [18, 64], other vole-associated hantaviruses such as PUUV have been reported to reach a molecular prevalence of 60 %-100 % in reservoir host populations [65, 66]. The TULV seroprevalence of no more than 19 % observed here is much lower than antibody prevalence of up to 100 % reported for other Cricetidae- and Murinae-associated hantaviruses [66–71].

Our data suggest that – once in the common vole population – TULV seems to persist for months if not years. Persistence of hantaviruses in vole populations over several years has been shown for PUUV in bank voles. Distinct virus types were detectable over several years, independent of population density and virus prevalence [66]. Social behaviour, such as forming of colonies and kin clustering in winter, might contribute substantially to transmission and virus persistence, especially between closely related animals [72]. Our investigations have shown initial evidence of long-lasting presence of TULV in vole populations. Future systematic rodent-monitoring studies are needed to study potential intrinsic and extrinsic effects on TULV prevalence. Further studies should consider the potential oscillation of TULV prevalence in its reservoir according to season and long-term population dynamics [see ref. 66].

Discrepancy between serological and RT-PCR detection of TULV in the reservoir

This study shows a significant difference between seropositivity (49/670, 7.3 %) and molecular detection of TULV in common voles (105/685, 15.3 %) ($\chi^2 = 20.812$; df = 1; $p < 0.001$). One reason for this discrepancy might be a large number of acute TULV infections among common voles where no antibody response has been induced so far. This might be true in times of a high frequency of TULV transmission, perhaps during the peaks of the vole population. Alternatively, TULV might induce only a weak antibody response in the host, possibly due to an early innate immune response, as has been reported for *in vitro* investigations on the TULV-related PHV [73]. Furthermore, using a recombinant N protein originating from TULV strain Moravia for detection of other TULV strains in the ELISA might have caused a lower sensitivity of the IgG ELISA. Our sequence analysis (aa 225-307) including the hypervariable region of the N proteins [18] of members of TULV clades I to IV revealed sequence differences of up to 10.6 % at the amino acid level to the N protein of prototype strain Moravia (Supplementary Table 3). Additionally, the sensitivity of the ELISA might be influenced by the selection of the secondary antibody and setting of the cutoff value.

For nine adult common voles (weight, 18-36 g) TULV-reactive antibodies were found, but no viral nucleic acid, although TULV RNA was detected in other individuals from the same trapping sites and season. The detection of IgG antibodies in the absence of viral RNA may indicate virus clearance instead of a persistent infection. Similar

results were obtained in a study of PUUV in bank voles trapped in northern Sweden and for SNV in wild deer mice (*Peromyscus maniculatus*) [74, 75]. Furthermore, some studies of SEOV have suggested that, depending on the age of the reservoir upon infection, a persistent infection may not always be established [76]. Alternatively, this could be a sign of viral RNA load fluctuation during the course of infection.

In summary, our study showed a strong discrepancy between RT-PCR and serological detection of TULV infection in its reservoir. Future serological studies using antigens from different TULV strains should allow their role in the observed lower sensitivity of the ELISA compared to the RT-PCR to be tested. In addition, in-depth studies are needed to clarify the role of the rodent immune system in TULV infection and possible age effects.

Phylogeography of TULV and its reservoir

The detection of 79 novel TULV sequences from a large geographic area provided detailed insights into the high sequence variability and genetic structuring of TULV in Central Europe. Our study also allows an initial comparison of the phylogeography of the virus and its reservoir rodent host. In cluster II, the sequence of prototype strain Lodz from Poland is flanked by TULV sequences originating from common voles of the Eastern lineage, mostly found in countries located east of Germany, and four individuals of the Central lineage trapped at the eastern German border where Central and Eastern vole lineages meet [39, 40, 77–79]. In addition, our study shows the existence of distinct genetic clusters of TULV in close proximity to each other. This is consistent with the genetic isolation of common vole populations, even on a small geographic scale [40, 80, 81], and may indicate processes of virus-host adaptation.

In conclusion, this study shows that TULV is widespread in Central European common vole populations. TULV RNA was more frequently found in common voles than in field and water voles, confirming the common vole as the reservoir host and suggesting that infection of other vole species is most likely due to spillover. We readily detected TULV RNA in voles at different sites, but we were less successful in detecting specific antibodies. Although this could be a methodological problem, mechanisms leading to a reduced adaptive immune response cannot be excluded, and this provides an interesting target for further studies. The overall prevalence of TULV was not nearly as high as reported for the related PUUV in its bank vole reservoir. For further assessment of the potential involvement of population dynamics on TULV prevalence additional studies are needed. These monitoring studies would also have to consider potential consequences of TULV infection on the fitness of the vole reservoir. The initial finding of large-scale associations of some TULV clades with different evolutionary lineages of common voles indicates the need for future studies in the contact areas to study potential (co-) evolutionary processes in more detail.

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Supplementary Table 1 Results of vole trappings and of TULV RT-PCR and ELISA investigations for each trapping site.

Country	# in map	Federal state	Trapping site	Individuals trapped			number of TULV positive animals								
							Marv			Magr			Arv		
				Marv	Magr	Arv	Serology (postive/ total analyzed)	RT-PCR (positive/ total analyzed)	**total positive/ total analyzed**	Serology (positive/ total analyzed)	RT-PCR (positive/ total analyzed)	**total positive/ total analyzed)**	Serology (positive/ total analyzed)	RT-PCR (positive/ total analyzed)	**total positive/ total analyzed)**
Germany	01	**MP**	**Frätow**	1	1	0	1/1	1/1	**1/1**	1/1	0/1	**1/1**	-	-	-
	02	**MP**	**Horst/Jeeser**	32	3	0	6/31	12/32	**14/32**	0/3	0/3	**0/3**	-	-	-
	03	**MP**	**Lühmannsdorf**	6	0	0	1/6	1/6	**1/6**	-	-	-	-	-	-
	04	**MP**	**Tremt**	7	0	0	1/7	1/7	**1/7**	-	-	-	-	-	-
	05	SH	Probsteierhagen	1	0	0	0/1	0/1	**0/1**	-	-	-	-	-	-
	06	**SH**	**Schrevendorf**	6	1	0	0/6	2/6	**2/6**	0/1	0/1	**0/1**	-	-	-
	07	**LS**	**Göttingen**	1	0	0	1/1	1/1	**1/1**	-	-	-	-	-	-
	08	LS	Wardenburg	2	1	0	0/2	0/2	**0/2**	0/1	0/1	**0/1**	-	-	-
	09	**LS**	**Westergellersen**	15	0	0	0/15	2/15	**2/15**	-	-	-	-	-	-
	10	**LS**	**Wolbrechtshausen**	2	0	0	2/2	2/2	**2/2**	-	-	-	-	-	-
	11	ST	Calvörde	2	19	0	0/2	0/2	**0/2**	0/19	0/19	**0/19**	-	-	-
	12	ST	Halle	1	0	0	n.d.	0/1	**0/1**	-	-	-	-	-	-
	13	**ST**	**Hausneindorf**	19	0	0	1/19	3/19	**3/19**	-	-	-	-	-	-
	14	**ST**	**Morl**	50	0	0	1/50	3/50	**3/50**	-	-	-	-	-	-
	15	**ST**	**Calvörde-Walbeck**	0	1	0	-	-	-	1/1	1/1	**1/1**	-	-	-
	16	**BB**	**Altdöbern, Crahnsdorf**	2	0	0	0/2	2/2	**2/2**	-	-	-	-	-	-
	17	**BB**	**Lug**	10	0	0	0/10	1/10	**1/10**	-	-	-	-	-	-
	18	**BB**	**Biebersdorf**	2	4	0	0/2	1/2	**1/2**	1/2	3/4	**3/4**	-	-	-
	19	BB	Bad Wilsnack/ Bendelin	2	1	0	0/2	0/2	**0/2**	0/1	0/1	**0/1**	-	-	-

20	**BB**	**Groß Schönebeck**	5	0	0	0/5	1/5	**1/5**	-	-	-	-	-	-
21	**BB**	**Hühnerwasser**	3	0	0	3/3	3/3	**3/3**	-	-	-	-	-	-
22	**BB**	**Muckrow**	20	0	0	3/20	6/20	**7/20**	-	-	-	-	-	-
23	BB	Raben-Marzehns	0	7	0	-	-	-	0/7	0/7	**0/7**	-	-	-
24	**BE**	**Berlin-Gatow**	7	2	0	0/5	1/7	**1/7**	0/2	0/2	**0/2**	-	-	-
25	BE	Berlin-Steglitz	4	0	0	0/4	0/4	**0/4**	-	-	-	-	-	-
26	SN	Colmnitz	40	0	7	0/40	0/40	**0/40**	-	-	-	0/7	0/7	**0/7**
27	**SN**	**Cunnersdorf**	32	0	0	2/29	4/32	**4/32**	-	-	-	-	-	-
28	SN	Eckhardtsberg	1	0	0	0/1	0/1	**0/1**	-	-	-	-	-	-
29	SN	Guttau	1	0	0	0/10	0/1	**0/1**	-	-	-	-	-	-
30	**SN**	**Königshain**	1	0	0	1/1	1/1	**1/1**	-	-	-	-	-	-
31	SN	**(Leipgen)**	0	1	0	-	-	-	**1/1**	0/1	**1/1**	-	-	-
32	SN	**Lohsa**	3	0	0	1/3	2/3	**2/3**	-	-	-	-	-	-
33	SN	**(Nassau)**	0	6	0	-	-	-	1/6	0/6	**1/6**	-	-	-
34	SN	Wermsdorf, Hubertusburg	0	2	0	-	-	-	0/2	0/2	**0/2**	-	-	-
35	SN	Wermsdorf, Collm	2	1	0	2/2	0/2	**0/2**	0/1	0/1	**0/1**	-	-	-
36	SN	Rehefeld-Zaunhaus	0	17	0	-	-	-	0/16	0/17	**0/17**	-	-	-
37	SN	Sohland am Rotstein	1	0	0	0/1	0/1	**0/1**	-	-	-	-	-	-
38	**TH**	**Gotha-Siebleben**	4	0	0	4/4	3/4	**4/4**	-	-	-	-	-	-
39	**TH**	**Gotha-West**	37	44	0	6/37	9/37	**9/37**	7/44	6/44	**9/44**	-	-	-
40	**TH**	**Schaderode**	143	0	0	5/143	13/143	**13/143**	-	-	-	-	-	-
41	**TH**	**Treben (1) 228a2**	93	21	0	2/93	3/93	**5/93**	0/21	0/21	**0/21**	-	-	-
42	**TH**	**Treben (2) 455z1**	4	39	0	0/4	0/4	**0/4**	1/39	1/39	**2/39**	-	-	-
43	TH	Treben (3) 402z2	0	10	0	-	-	-	0/10	0/10	**0/10**	-	-	-
44	**BY**	**Balzhausen**	11	1	1	1/10	2/11	**3/11**	1/1	0/1	**1/1**	0/1	1/1	**1/1**
45	BY	Buchloe	6	0	0	0/6	0/6	**0/6**	-	-	-	-	-	-
46	BY	Dillingen	0	2	0	-	-	-	0/2	0/2	**0/2**	-	-	-
47	BY	Ergoldbach	0	4	0	-	-	-	0/4	0/4	**0/4**	-	-	-

	48	BY	Freising	0	5	0	-	-	-	0/5	0/5	**0/5**	-	-	-
	49	BY	Geisfeld	0	8	0	-	-	-	0/8	0/8	**0/8**	-	-	-
	50	BY	Gräfenbuch	0	2	0	-	-	-	0/2	0/2	**0/2**	-	-	-
	51	**BY**	**(Grimoldsried)**	0	2	17	-	-	-	0/2	0/2	**0/2**	2/16	0/17	**2/17**
	52	BY	Hammeröd	0	2	0	-	-	-	0/2	0/2	**0/2**	-	-	-
	53	BY	Hiltenfingen	1	0	0	0/1	0/1	**0/1**	-	-	-	-	-	-
	54	BY	Herrieden	0	1	0	-	-	-	0/1	0/1	**0/1**	-	-	-
	55	BY	Kaisheim	0	3	0	-	-	-	0/3	0/3	**0/3**	-	-	-
	56	BY	Kammlach	0	15	0	-	-	-	0/15	0/15	**0/15**	-	-	-
	57	**BY**	**Kirchheim**	22	0	0	1/20	7/21	**8/22**	-	-	-	-	-	-
	58	BY	Königsbrunn	1	0	0	0/1	0/1	**0/1**	-	-	-	-	-	-
	59	**BY**	**Lauterhofen**	1	0	0	0/1	1/1	**1/1**	-	-	-	-	-	-
	60	BY	Lehrberg	3	3	0	0/3	0/3	**0/3**	0/3	0/3	**0/3**	-	-	-
	61	BY	Obermedlingen	0	2	0	-	-	-	0/2	0/2	**0/2**	-	-	-
	62	BY	Rain am Lech	0	6	0	-	-	-	0/6	0/6	**0/6**	-	-	-
	63	BY	Strullendorf	0	1	0	-	-	-	0/1	0/1	**0/1**	-	-	-
	64	BW	Weissach	2	0	0	0/2	0/2	**0/2**	-	-	-	-	-	-
	65	**HE**	**Mücke-Merlau**	7	9	1	1/7	2/7	**2/7**	4/9	2/9	**4/9**	0/1	0/1	**0/1**
	66	**HE**	**Griesheim**	1	0	0	n.d.	1/1	**1/1**	-	-	-	-	-	-
	67	**NW**	**Rüthen**	4	0	0	1/4	2/4	**2/4**	-	-	-	-	-	-
	68	**NW**	**Soest**	9	0	0	0/9	1/9	**1/9**	-	-	-	-	-	-
	69	**NW**	**Theesen**	1	1	0	1/1	1/1	**1/1**	0/1	0/1	**0/1**	-	-	-
	70	**NW**	**Warburg**	22	1	0	0/22	2/22	**2/22**	0/1	0/1	**0/1**	-	-	-
	71	**RP**	**Koblenz**	1	0	0	n.d.	1/1	**1/1**	-	-	-	-	-	-
Luxem-bourg	72		Cruchten	0	0	1	-	-	-	-	-	-	0/1	0/1	**0/1**
	73		**Schlindermanderscheid**	6	0	3	2/6	2/6	**3/6**	-	-	-	0/3	0/3	**0/3**
France	74	AL	Adamswiller	4	0	0	0/4	0/4	**0/4**	-	-	-	-	-	-
	75	AL	**Berg**	4	0	0	0/4	3/4	**3/4**	-	-	-	-	-	-
	76	AL	Puberg	2	0	0	0/2	0/2	**0/2**	-	-	-	-	-	-

	77	AL	Struth	10	0	0	0/10	0/10	**0/10**	-	-	-	-	-	-
	78	Mpy	**Millau**	4	0	0	-	2/4	**2/4**	-	-	-	-	-	-
	79	AL	**Tieffenbach**	2	0	0	0/2	1*/2	**1*/2**	-	-	-	-	-	-

Bold = trapping sites with RNA positive animals, (bold) = trapping sites with animals positive in serology only, Marv *Microtus arvalis*, Magr *Microtus agrestis*, Arv *Arvicola* spec., abbreviations federal states/administrative districts: AL Alsace, BB Brandenburg, BE Berlin, BW Baden-Wuerttemberg, BY Bavaria, HE Hesse, MP Mecklenburg Western-Pomerania, LS Lower Saxony, Mpy Midi-Pyrénées, NW North Rhine-Westphalia, RP Rhineland-Palatinate, SH Schleswig-Holstein, SN Saxony, ST Saxony-Anhalt, TH Thuringia

Supplementary Table 2 Results of TULV RT-PCR and ELISA for animals collected repeatedly at sites with animals being positive at least at one time point.

Trapping site	Federal state	Trapping date	Marv				Magr				Arv			
			n trapped	Serology (positive/ total n tested)	RT-PCR (positive/ total n tested)	Total positive/ total tested	n trapped	Serology (positive/ total n tested)	RT-PCR (positive/ total n tested)	Total positive/total tested	n trapped	Serology (positive/ total n tested)	RT-PCR (positive/ total n tested)	total positive/ total tested
01	**MP**	**October 2007**	**1**	**1/1**	**1/1**	**1/1**	-	-	-	-	-	-	-	-
		November 2007	-	-	-	-	**1**	**1/1**	**0/1**	**1/1**	-	-	-	-
06	**SH**	September 2009	1	0/1	0/1	0/1	-	-	-	-	-	-	-	-
		September 2010	-	-	-	-	1	0/1	0/1	0/1	-	-	-	-
		October 2012	**3**	**0/3**	**2/3**	**2/3**	-	-	-	-	-	-	-	-
		November 2012	1	0/1	0/1	0/1	-	-	-	-	-	-	-	-
		Dezember 2012	1	0/1	0/1	0/1	-	-	-	-	-	-	-	-
09	**LS**	**April 2008**	**2**	**0/2**	**1/2**	**1/2**	-	-	-	-	-	-	-	-
		September 2008	**13**	**0/13**	**1/13**	**1/13**	-	-	-	-	-	-	-	-
10	**LS**	**September 2008**	**1**	**1/1**	**1/1**	**1/1**	-	-	-	-	-	-	-	-
		October 2008	**1**	**1/1**	**1/1**	**1/1**	-	-	-	-	-	-	-	-
24	**BE**	**April 2011**	**2**	-	**1/2**	**1/2**	-	-	-	-	-	-	-	-
		August 2011	2	0/2	0/2	0/2	-	-	-	-	-	-	-	-
		September 2011	2	0/2	0/2	0/2	-	-	-	-	-	-	-	-
		October 2011	2	0/2	0/2	0/2	-	-	-	-	-	-	-	-
		November 2011	1	0/1	0/1	0/1	-	-	-	-	-	-	-	-
27	**SN**	**November 2006**	**17**	**1/17**	**3/17**	**3/17**	-	-	-	-	-	-	-	-
		December 2006	**15**	**1/12**	**1/12**	**1/12**	-	-	-	-	-	-	-	-
39	**TH**	**September 2005**	**19**	**4/15**	**5/15**	**5/15**	**19**	**3/19**	**5/19**	**5/19**	-	-	-	-
		June 2007	-	-	-	-	2	0/2	0/2	0/2	-	-	-	-
		September 2007	**8**	**1/8**	**2/8**	**2/8**	**12**	**2/12**	**1/12**	**2/12**	-	-	-	-
		June 2008	-	-	-	-	2	0/2	0/2	0/2	-	-	-	-

		October 2008	**10**	**1/10**	**2/10**	**2/10**	**9**	**2/9**	0/9	**2/9**	-	-	-	-
40	**TH**	**December 2006**	**5**	0/5	**1/5**	**1/5**	-	-	-	-	-	-	-	-
		October 2009	**110**	**4/110**	**11/110**	**11/110**	-	-	-	-	-	-	-	-
		December 2009	28	0/28	0/28	0/28	-	-	-	-	-	-	-	-
41	**TH**	**June 2009**	**4**	**0/4**	**2/4**	**2/4**	-	-	-	-	-	-	-	-
		September 2009	**25**	**0/25**	**1/25**	**1/25**	1	0/1	0/1	0/1	-	-	-	-
		October 2009	**30**	**1/30**	**0/30**	**1/30**	20	0/20	0/20	0/20	-	-	-	-
		November 2009	34	0/34	0/34	0/34	-	-	-	-	-	-	-	-
42	**TH**	May 2009	-	-	-	-	2	0/2	0/2	0/2	-	-	-	-
		July 2009	-	-	-	-	3	0/3	0/3	0/3	-	-	-	-
		September 2009	**4**	0/4	0/4	0/4	**12**	**2/12**	**1/12**	**2/12**	-	-	-	-
		November 2009	-	-	-	-	22	0/22	0/22	0/22	-	-	-	-
44	**BY**	June 2012	**1**	0/1	0/1	0/1	-	-	-	-	-	-	-	-
		July 2012	**8**	**1/6**	**2/8**	**3/8**	**1**	**1/1**	**0/1**	**1/1**	**1**	**0/1**	**1/1**	**1/1**
		August 2012	**2**	**0/2**	**1/2**	**1/2**	-	-	-	-	-	-	-	-
51	**BY**	**June 2012**	-	-	-	-	-	-	-	-	**7**	**1/7**	**0/7**	**1/7**
		July 2012	-	-	-	-	2	0/2	0/2	0/2	**7**	**1/7**	**0/7**	**1/7**
		August 2012	-	-	-	-	-	-	-	-	3	0/3	0/3	0/3
		September 2012	-	-	-	-	-	-	-	-	1	0/1	0/1	0/1
57	**BY**	June 2012	1	-	0/1	0/1	-	-	-	-	-	-	-	-
		July 2012	**5**	**0/5**	**1/5**	**1/5**	-	-	-	-	-	-	-	-
		August 2012	**10**	**1/10**	**2/10**	**3/10**	-	-	-	-	-	-	-	-
		September 2012	**6**	**0/6**	**4/6**	**4/6**	-	-	-	-	-	-	-	-
65	**HE**	**August 2007**	**2**	**0/2**	**1/2**	**1/2**	**3**	**2/3**	**0/3**	**2/3**		-	-	-
		September 2007	**5**	**1/5**	**1/5**	**1/5**	**4**	**2/4**	**2/4**	**2/4**	1	0/1	0/1	0/1
		October 2007	-	-	-	-	2	0/2	0/2	0/2	-	-	-	-
75	**AL**	**June 2012**	**1**	**0/1**	**1/1**	**1/1**	-	-	-	-	-	-	-	-
		August 2012	**2**	**0/2**	**1/2**	**1/2**	-	-	-	-	-	-	-	-
		September 2012	**1**	**0/1**	**1/1**	**1/1**	-	-	-	-	-	-	-	-

Marv *Microtus arvalis*, Magr *Microtus agrestis*, Arv *Arvicola* spec., AL Alsace, BY Bavaria, BE Berlin, HE Hesse, LS Lower Saxony , SN Saxony, TH Thuringia, bold = positive animals detected; * no sequence

Supplementary Table 3 Mean nucleocapsid protein amino acid (aa) and S segment nucleotide (nt) sequence identity between TULV prototype strain Moravia (acc.no NC_005227) and the sequence clades in Europe.

	Ia	Ib	II	III	IV
aa	90.3%	89.4%	92.1%	95.4%	99.1%
nt	80%	80.4%	81.7%	84.5%	94.6%

Supplementary Table 4 Accession numbers of all newly generated TULV sequences

Trapping site	Sequence	Acc.no
01	07_1457_Frae_Marv	KU139527
02	09_2324_HT_Marv	KU139528
02	09_2375_HT_Marv	KU139529
02	10_0023_HT_Marv	KU139530
02	10_0029_HT_Marv	KU139531
03	08_0092_Lueh_Marv	KU139532
04	07_0862_Trem_Marv	KU139533
06	13_0772_Schrev_Marv	KU139534
06	13_0784_Schrev_Marv	KU139535
07	11_1373_Goett_Marv	KU139536
09	08_0639_WG_Marv	KU139537
09	08_1045_WG_Marv	KU139538
10	11_1429_Wolbr_Marv	KU139539
10	11_1433_Wolbr_Marv	KU139540
13	08_0789_Hausn_Marv	KU139541
13	08_0802_Hausn_Marv	KU139542
14	08_0848_Morl_Marv	KU139543
14	08_0849_Morl_Marv	KU139544
14	08_0894_Morl_Marv	KU139545
15	07_0081_Walb_Magr	KU139546
16	10_1622_Crah_Marv	KU139547
17	10_1625_Lug_Marv	KU139548
18	09_1886_Bieb_Marv	KU139549
18	09_1901_Bieb_Magr	KU139550
18	09_1905_Bieb_Magr	KU139551
18	09_1912_Bieb_Magr	KU139552
20	09_1477_GroSch_Marv	KU139553
21	09_1648_Hueh_Marv	KU139554
21	09_1657_Hueh_Marv	KU139555
21	09_1669_Hueh_Marv	KU139556
22	10_1661_Muck_Marv	KU139557
24	D34_Gato_Marv	KU139558
27	09_0972_Cunn_Marv	KU139559
27	09_1000_Cunn_Marv	KU139560
30	09_0928_Koen_Marv	KU139561
32	09_1917_Loh_Marv	KU139562
32	09_1939_Loh_Marv	KU139563
38	08_0534_Siebl_Marv	KU139564
38	08_0538_Siebl_Marv	KU139565
39	08_0350_Goth_Marv	KU139566
39	08_0352_Goth_Marv	KU139567
39	08_0356_Goth_Marv	KU139568
39	08_0362_Goth_Marv	KU139569
39	08_0545_Goth_Marv	KU139570
39	12_0492_Goth_Magr	KU139571
39	12_0526_Goth_Magr	KU139572

40	10_1533_Scha_Marv	KU139573
40	12_1068_Scha_Marv	KU139574
40	12_1121_Scha_Marv	KU139575
41	10_0905_Tre_Marv	KU139576
41	10_0908_Tre_Marv	KU139577
41	10_0932_Tre_Marv	KU139578
42	10_1188_Tre_Magr	KU139579
44	S1368_12_Balz_Marv	KU139580
44	S1449_12_Balz_Arv	KU139581
44	S1450_12_Balz_Marv	KU139582
57	S729_13_Kirch_Marv	KU139583
57	S736_13_Kirch_Marv	KU139584
57	S740_13_Kirch_Marv	KU139585
57	S747_13_Kirch_Marv	KU139586
57	S750_13_Kirch_Marv	KU139587
57	S751_13_Kirch_Marv	KU139588
59	S596_13_Laut_Marv	KU139589
65	08_0205_MueM_Magr	KU139590
65	08_0216_MueM_Marv	KU139591
65	08_0218_MueM_Magr	KU139592
65	08_0236_MueM_Marv	KU139593
66	VIII_3_Gries_Marv	KU139594
67	10_0183_Ruet_Marv	KU139595
67	10_0185_Ruet_Marv	KU139596
68	10_0215_Soes_Marv	KU139597
69	08_1033_Thee_Marv	KU139598
70	08_0260_Warb_Marv	KU139599
70	08_0277_Warb_Marv	KU139600
71	E4_Kobl_Marv	KU139601
73	S666_13_Lux_Marv	KU139602
73	S667_13_Lux_Marv	KU139603
75	12_3019_Elsa_Marv	KU139604
78	73_Mill123	KU139605

Supplementary Table 5 Accession numbers of all newly generated *cytochrome b* sequences of *Microtus arvalis*

Sequence	Acc.no	mtDNA lineage
07_1457_MP	KU139696	Central
09_2324_MP	KU139697	Central
09_2375_MP	KU139698	Central
10_0029_MP	KU139699	Central
09_0092_MP	KU139700	Central
07_0862_MP	KU139701	Central
13_0772_SH	KU139702	Central
13_0779_SH	KU139703	Central
13_1373_Goett	KU139704	Central
08_0658_LS	KU139705	Central
08_0639_NI	KU139706	Central
08_1045_NI	KU139707	Central
07_0037_ST	KU139708	Central
11_1429_NI	KU139710	Central
11_1433_NI	KU139711	Central
08_0828_SN	KU139709	Central
08_0789_ST	KU139712	Central
08_0802_ST	KU139713	Central
08_0848_ST	KU139714	Central
08_0849_ST	KU139715	Central
08_0894_ST	KU139716	Central
08_0906_ST	KU139717	Central
10_1622_BB	KU139718	Central
10_1625_BB	KU139719	Central
09_1880_BB	KU139720	Eastern
09_1886_BB	KU139721	Eastern
09_1477_BB	KU139722	Central
09_1648_BB	KU139723	Central
09_1657_BB	KU139724	Central
09_1669_BB	KU139725	Central
10_1661_BB	KU139726	Eastern
D34_BE	KU139727	Central
13_0807_SN	KU139728	Central
13_0808_SN	KU139729	Central
13_0809_SN	KU139730	Central
09_0966_SN	KU139731	Central
09_0967_SN	KU139732	Central
09_0969_SN	KU139733	Central
09_0970_SN	KU139734	Eastern
09_0971_SN	KU139735	Central
09_0972_SN	KU139736	Eastern
09_0973_SN	KU139737	Eastern
09_0974_SN	KU139738	Central
09_0975_SN	KU139739	Central
09_0976_SN	KU139740	Central

09_0977_SN	KU139741	Central
09_0979_SN	KU139742	Central
09_0980_SN	KU139743	Central
09_0981_SN	KU139744	Central
09_0982_SN	KU139745	Central
09_0983_SN	KU139746	Eastern
09_0984_SN	KU139747	Eastern
09_0985_SN	KU139748	Central
09_0986_SN	KU139749	Central
09_0987_SN	KU139750	Central
09_0988_SN	KU139751	Central
09_0989_SN	KU139752	Central
09_0991_SN	KU139753	Central
09_0996_SN	KU139754	Central
09_1000_SN	KU139755	Central
09_1001_SN	KU139756	Central
09_1002_SN	KU139757	Central
09_1003_SN	KU139758	Eastern
09_1004_SN	KU139759	Central
09_0867_SN	KU139760	Eastern
09_0917_SN	KU139761	Eastern
09_0928_SN	KU139762	Eastern
09_1917_SN	KU139763	Eastern
09_1939_SN	KU139764	Eastern
09_1948_SN	KU139765	Eastern
09_1016_SN	KU139766	Central
09_1018_SN	KU139767	Central
08_0887_SN	KU139768	Eastern
08_0525_TH	KU139769	Central
08_0538_TH	KU139770	Central
08_0350_TH	KU139772	Central
08_0352_TH	KU139773	Central
08_0356_TH	KU139774	Central
08_0362_TH	KU139775	Central
08_0545_TH	KU139771	Central
10_1533_TH	KU139776	Central
12_1068_TH	KU139777	Central
11_1121_TH	KU139778	Central
10_0905_TH	KU139779	Central
10_0908_TH	KU139780	Central
10_0932_TH	KU139781	Central
10_1151_TH	KU139782	Central
S1368_12_BY	KU139783	Central
09_1370_BY	KU139784	Central
S729_13_BY	KU139785	Central
S736_13_BY	KU139786	Central
S740_13_BY	KU139787	Central

S747_13_BY	KU139788	Central
S750_13_BY	KU139789	Central
S596_13_BY	KU139790	Central
09_1387_BY	KU139791	Central
10_1938_BW	KU139792	Central
08_0216_HE	KU139793	Central
08_0236_HE	KU139794	Central
VIII3_HE	KU139795	Central
10_0183_NW	KU139796	Central
10_0215_NW	KU139797	Central
08_1033_NW	KU139798	Central
08_0260_NW	KU139799	Central
08_0277_NW	KU139800	Central
S666_13_Lux	KU139801	Western
S667_13_Lux	KU139802	Western
12_3005_FR	KU139803	Western
12_3015_FR	KU139804	Western
12_3008_FR	KU139805	Western
12_3019_FR	KU139806	Western
12_2999_FR	KU139807	Western
12_3000_FR	KU139808	Western
12_3010_FR	KU139809	Western
12_3025_FR	KU139810	Western
Mill076	KU139811	Western
Mill109	KU139812	Western
Mill123	KU139813	Western
Mill124	KU139814	Western
12_3007_FR	KU139815	Western
12_3017_FR	KU139816	Western

(II) SPATIAL AND TEMPORAL DYNAMICS AND MOLECULAR EVOLUTION OF *TULA ORTHOHANTAVIRUS* IN GERMAN VOLE POPULATIONS

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Spatial and Temporal Dynamics and Molecular Evolution of *Tula orthohantavirus* in German Vole Populations

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Abstract: Tula orthohantavirus (TULV) is a rodent-borne hantavirus with broad geographical distribution in Europe. Its major reservoir is the common vole (*Microtus arvalis*), but TULV has also been detected in closely related vole species. Given the large distributional range and high amplitude population dynamics of common voles, this host–pathogen complex presents an ideal system to study the complex mechanisms of pathogen transmission in a wild rodent reservoir. We investigated the dynamics of TULV prevalence and the subsequent potential effects on the molecular evolution of TULV in common voles of the Central evolutionary lineage. Rodents were trapped for three years in four regions of Germany and samples were analyzed for the presence of TULV-reactive antibodies and TULV RNA with subsequent sequence determination. The results show that individual (sex) and population-level factors (abundance) of hosts were significant predictors of local TULV dynamics. At the large geographic scale, different phylogenetic TULV clades and an overall isolation-by-distance pattern in virus sequences were detected, while at the small scale (<4 km) this depended on the study area. In combination with an overall delayed density dependence, our results highlight that frequent, localized bottleneck events for the common vole and TULV do occur and can be offset by local recolonization dynamics.

Keywords: rodents; hantavirus; monitoring; population dynamics; common vole; field vole; water vole; phylogeny; molecular evolution

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1. Introduction

Tula orthohantavirus (TULV) is a European hantavirus that was initially discovered in the common vole (*Microtus arvalis*) and the sibling vole (*M. levis*, previously *M. rossiaemeridionalis*) [1,2]. In addition, TULV was detected in other vole species, such as field vole (*M. agrestis*), European pine vole (*M. subterraneus*), narrow-headed vole (*M. gregalis*), Major's pine vole (*Microtus majori*) and water vole (*Arvicola* spp.) [3–9]. These multiple molecular surveys confirmed the role of the common vole as the major reservoir, with a usually low to medium prevalence [9]. Infections in voles other than the common vole seem to reflect spillover infections [9], although in rare cases the field vole may represent an alternative reservoir [6]. TULV-related viruses have been identified in various other *Microtus* species in Eurasia [10–14].

TULV contains a trisegmented RNA genome of negative polarity with the small (S) segment encoding the nucleocapsid (N) protein, but also a putative non-structural (NSs) protein with interferon antagonist properties [15]. The medium (M) segment encodes a glycoprotein precursor that is co-translationally cleaved into two glycoproteins, whereas the large (L) segment encodes an RNA-dependent RNA polymerase with several enzymatic functions [16]. Based on nucleotide sequences, genetically divergent TULV clades have been identified that partially reflect the association to evolutionary lineages in the common vole in Central Europe [9,17,18].

TULV is commonly described as non-pathogenic to humans, with very few cases of human infections or of seroconversion being reported [16,19–22]. TULV-reactive antibodies have been detected in forestry workers in Brandenburg, eastern Germany [20]. A hospitalized patient with symptoms of hemorrhagic fever with renal syndrome from the same federal state was shown to have neutralizing antibodies specific for TULV [23]. Further, in an immune-compromised patient from the Czech Republic TULV RNA was detected [21]. Recently, a human TULV infection with acute kidney injury was detected in Germany [24].

The common vole is widely distributed in Central Europe and as the most abundant mammal species it predominately inhabits natural and agricultural grassland habitats [25]. Apart from seasonal changes in population size, this species is known to undergo multi-annual fluctuation (outbreaks) [26] that are correlated to weather conditions [27,28] and habitat factors [29]. Outbreak maxima exceed 2000 individuals/ha [30] and are observed about every 3–5 years [31]. While large-scale, synchronous outbreaks have been reported for Europe [32], cyclicity itself does not appear to be synchronous over the whole distribution range. For many rodent-borne pathogens, reservoir density-dependent transmission is a key feature of pathogen circulation as increasing host density theoretically promotes human incidence [33]. In addition, there is evidence of a strong interaction between host population dynamics, hantavirus circulation and subsequent molecular evolution. For Puumala orthohantavirus (PUUV) transmitted by bank voles (*Myodes glareolus*, formerly *Clethrionomys glareolus*) this includes seasonal and annual density dependence of pathogen circulation within the rodent host [34–37]. To date, there is little known about similar interactions in common vole populations and TULV. Here, we present the results of a longitudinal study in four regions of Germany assessing TULV prevalence and nucleotide sequence evolution in fluctuating common vole populations. We hypothesize that within common vole populations TULV prevalence is positively correlated with abundance. Additionally, we hypothesize that TULV sequence similarity reflects the association with evolutionary lineages of the common voles and is negatively correlated to increasing spatial distance between the sites, indicating that factors limiting dispersal between populations are key drivers of local molecular virus evolution.

2. Materials and Methods

2.1. Rodent Trapping and Sample Collection

Voles were collected during 2010 to 2013 in spring, summer and autumn in four study areas in Germany: Jeeser (54°9.75′ N, 13°15.55′ E, Mecklenburg-Western Pomerania), Gotha (50°57.38′ N, 10°39.13′ E, Thuringia), Billerbeck (51°59.63′ N, 7°18.99′ E, North Rhine-Westphalia) and Weissach (48°49.88′ N, 8°57.71′ E, Baden-Wuerttemberg) (Figure 1). Trapping was conducted on permanent grasslands used mainly for silage production. Within each study area, three replicate sites were established in close proximity (<4 km), and within each site both live and snap trapping were performed (around 200 m apart). During trapping specific biosafety measures were followed, including wearing protective clothing, gloves and a FFP3 mask.

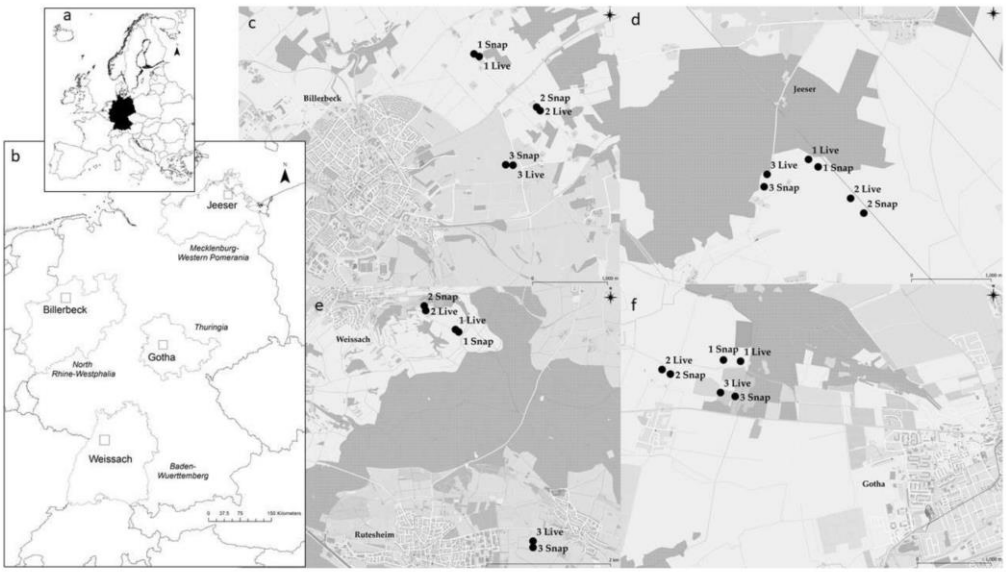

Figure 1. Map of the four study areas in Germany ((**a**), overview) and the corresponding federal states ((**b**), grey). In each area (Billerbeck (**c**), Jeeser (**d**), Weissach (**e**), Gotha (**f**)), trapping was conducted on three replicate sites (1, 2, 3) where live (Live) and snap (Snap) trapping was performed. Dark-grey areas present forests and light-grey areas are agricultural/grassland areas where the trapping was performed.

The snap trapping followed a standard protocol (see APHAEA standard protocol; http://www.aphaea.org/cards/species/voles, accessed on 12 October 2018). At each site, a grid of 7 × 7 traps with 10 m inter-trap distance was used and traps were baited with raisins. Rodent dissection and the collection of lung and other tissue samples followed previously established standard protocols [38]. The chest cavity was rinsed with 1 mL phosphate-buffered saline (PBS); the resulting chest cavity lavage (CCL) samples were used for detection of TULV-reactive antibodies. The dissection was performed within a BSL-3 containment dissection hall following standard hygiene and personal protection instructions.

Live trapping was conducted using the same general set-up with Ugglan live traps following procedures described previously [35]. In brief, traps were pre-baited for three days and checked twice a day for 2–3 consecutive days. Trapped animals were sexed and weighed using a 50 g spring scale (PESOLA AG®, Schindellegi, Switzerland). After species determination, voles were marked with a passive integrated transponder (PIT) tag (LUX-IDent s.r.o.®, Lanškroun, Czech Republic) for individual identification. Small ear pinna tissue samples were collected and stored in 80% ethanol. Blood samples (20–40 µL) were collected using the *Vena facialis* or the retro-orbital sinus and stored at −20 °C until analysis for TULV-reactive antibodies. After processing, animals were released at the point of capture. Animals found dead in live trapping were subjected to dissection as described above.

Relative abundance indices as individuals per 100 trapping nights (individuals/100TN) were calculated for both trapping methodologies (see Table S1). A comparison of abundance indices from live and snap trapping showed a significant positive linear correlation ($F = 183.8$, $p \leq 0.001$, $r^2 = 0.82$). Thus, we combined live and snap trapping data per site. This increased the number of sites where TULV prevalence could be calculated, even during years/seasons with generally low host abundance.

2.2. Nucleic Acid Isolation

The RNA extraction of lung tissue was performed using a modified QIAzol extraction protocol [7]. DNA was obtained from tissue samples using conventional chloroform DNA extraction or tissue lysis overnight using ear pinna or tail tissue samples [9,39].

2.3. Molecular Species and Sex Determination

Morphological species determination using a species determination key [40] was confirmed for all animals who tested positive by a mitochondrial cytochrome *b* (cyt *b*) gene-specific PCR [41]. In addition, for selected common voles, the mitochondrial DNA lineage in the species was determined as described before [9,42]. In case of missing morphological sex determination, sex was identified via PCR according to standard protocols [43,44].

2.4. TULV Detection

Detection of TULV-reactive antibodies in blood samples from live trapping as well as CCL samples from snap trapping with IgG ELISA followed previously published protocols using the yeast-expressed recombinant N protein of the TULV strain Moravia [6,20]. Hantavirus RT-PCR investigations of lung tissue samples from snap trapping followed previously described protocols for the PUUV/TULV S segment [45]. In addition, partial M and L segment sequences were determined after RT-PCR using the primers C1m (5′-CCAGCTGATTGCCCAGGGGTAG) and C2m (5′-CCTACTCCTGAGCCCCATGC; modified from [6]) and Han LF1 (5′-ATGTAYGTBAGTGCWGATGC) and Han LR1 (5′-AACCADT CWGTYCCRTCATC; [46]).

2.5. Sequence Determination and Phylogenetic Analyses

Sequence determination was performed by direct sequencing of RT-PCR products following a dideoxy-chain termination method using BigDye Terminator v1.1 kit (Applied Biosystems®, Darmstadt, Germany) and Genetic Analyser 3130 and 3130xl sequencing machines (Applied Biosystems®).

All generated sequences were subjected to a BLAST search-mediated comparison with sequences available in GenBank [47]. All TULV sequences were included in subsequent phylogenetic analysis. For common vole lineage analysis, three to four common voles from every trapping location were chosen for cyt *b* gene determination. Identical sequences were excluded from further analysis. Additional to the novel sequences obtained in this study, TULV sequences representative for the clades Central North (CEN.N), Eastern North (EST.N), Central South (CEN.S) and Eastern South (EST.S) were obtained from GenBank [47] and were labeled with accession numbers in Figure S1. The final datasets used for analysis contained 25 S segment sequences of 575 nucleotides (nt) length from the trapping sites Jeeser (n = 7) and Gotha (n = 8) and sequences of 572 nt length from the trapping sites Billerbeck (n = 3) and Weissach (n = 7), 21 M segment sequences of 618 nt length and 26 L segment sequences of 411 nt length for TULV and 14 sequences of 825 nt length from the cyt *b* gene of the common voles. Reference sequences for cyt *b* analysis were chosen according to [9].

Alignments were constructed in Bioedit (V7.2.3.) [48] using the Clustal W Multiple Alignment algorithm implemented in the program. Identical sequences were discarded from the alignment (see Table S6). The tree reconstructions were done via CIPRES [49] using partial S segment sequences of TULV (alignment length 549 nt, positions 406–951, counting according to TULV S segment, accession number NC_005227), partial M segment sequences of TULV (alignment length 348 nt, positions 2537–2884, counting according to TULV M segment, accession number NC_005228) and partial L segment sequences of TULV (alignment length 327 nt, positions 2983–3309, counting according to TULV L segment, accession number NC_005226).

Consensus phylogenetic trees of partial S, M and L segment sequences were generated by Bayesian analyses with 1×10^7 generations and a burn-in phase of 25%, and maximum-likelihood analyses were performed with 1000 bootstraps and 50% cut-off using the general

time-reversible (GTR) substitution model with invariant sites and a gamma-distributed shape parameter for both algorithms.

2.6. Isolation-by-Distance Analysis

We tested for isolation-by-distance patterns within and between the study regions based on S segment sequences and capture location information. Isolation-by-distance represents a positive association between genetic differences and spatial distance that establishes over time if dispersal occurs only at a local scale and the accumulation of mutations in viral strains is largely restricted to the local population [50]. Genetic distances between all pairs of sequences from the study sites were estimated in MEGA version X [51]. Spatial distances between the capture locations were determined with the *geosphere* package [52] in the R software [53]. Mantel tests were performed using the *ade4* package [54] and were used to assess statistical significance of the association between genetic and spatial distances.

2.7. Statistical Analysis

Differences in vole abundance as well as TULV seroprevalence between seasons, years and areas were analyzed by univariate analyses of variance (ANOVA) with subsequent post hoc tests (Tukey's HSD). Vole abundance or TULV seroprevalence were the dependent variables, and season, year and study area were fixed factors. Analyses were performed using $\alpha < 5\%$ as a level of significance.

A generalized linear mixed model (GLMM) with binomial distribution and a logit link function was used to statistically analyze the correlation of the common vole abundance index with TULV seroprevalence (level of significance $\alpha < 5\%$). The proportional response variable (two-vector variable) *TULV seroprevalence* was generated from the number of TULV-seropositive common voles and the number of TULV-seronegative common voles. The effects of the abundance index (direct effect) and the abundance index of the previous season (delayed effect), both in interaction with study area (factorial variable), were analyzed in two separate models. In each case, the trapping site nested in the study area was included as a random factor to account for the spatial and temporal design of the study. Analysis of deviance was performed to establish the overall significance of the categorical factors with more than two levels (study area). Overdispersion was checked using package *blmeco* [55] and function *dispersion glmer*. The number of paired observations of common vole abundance and TULV prevalence was $n = 43$. All analyses were done using R [53].

3. Results

3.1. Rodent Trapping

From 2010–2013 a total of 1487 common voles were caught (Table S1), and samples for TULV detection could be derived from 1304 individuals. Overall, 1062 common vole samples were derived from live trapping, and parallel snap trapping resulted in the collection of an additional 242 individuals (Table 1). In addition to common voles, a total of 180 field voles were trapped (Table S2).

Table 1. TULV seroprevalence in common vole populations in four German areas from 2010 to 2013. Seroprevalence (%) in spring, summer and autumn of each year was estimated for three replicate grassland sites per area based on live and snap trapping. Values for the number of positive tested individuals (positive/total) per season are given for all sites in each study area. Percentages were calculated only for sites with ≥5 tested individuals (otherwise NA = not applicable).

			Weissach		Jeeser		Billerbeck		Gotha		
Year	**Season**	**Site**	**Positive/ Total**	**%**	**Positive/ Total**	**%**	**Positive/ Total**	**%**	**Positive/ Total**	**%**	**Total %**
2010	Spring	1	1/6	16.7	0/9	0	0/0	NA	0/1	NA	2.8
		2	0/0	NA	0/1	NA	0/0	NA	0/2	NA	
		3	0/5	0	0/3	NA	0/0	NA	0/9	0	
	Summer	1	6/47	12.8	1/12	8.3	0/0	NA	4/18	22	14.1
		2	3/18	16.7	6/24	25	0/1	NA	2/27	7.4	
		3	0/13	0	16/84	19	0/5	0	0/20	0	
	Autumn	1	0/0	NA	0/15	0	0/2	NA	2/35	5.7	6.8
		2	0/2	NA	3/22	14	2/18	11	1/41	2.4	
		3	4/17	23.5	5/30	17	0/0	NA	7/49	14	
2011	Spring	1	0/0	NA	0/0	NA	0/0	NA	2/6	33	12.5
		2	0/0	NA	0/0	NA	0/0	NA	1/16	6.3	
		3	0/0	NA	1/2	NA	0/1	NA	0/7	0	
	Summer	1	0/16	0	0/0	NA	0/0	NA	0/24	0	0.0
		2	0/17	0	0/0	NA	0/0	NA	0/11	0	
		3	0/103	0	0/0	NA	0/1	NA	0/14	0	
	Autumn	1	0/4	NA	1/10	10	0/0	NA	1/33	3	7.4
		2	0/0	NA	0/14	0	0/4	NA	3/60	5	
		3	12/110	10.9	0/4	NA	0/9	0	4/35	11	
2012	Spring	1	0/0	NA	0/5	0	0/2	NA	2/16	13	11.4
		2	0/0	NA	0/0	NA	0/2	NA	0/0	NA	
		3	0/0	NA	0/2	NA	0/0	NA	2/8	25	
	Summer	1	2/21	9.5	0/8	0	0/0	NA	2/29	6.9	9.1
		2	0/2	NA	0/0	NA	0/0	NA	2/14	14	
		3	0/0	NA	0/1	NA	0/0	NA	7/35	20	
	Autumn	1	0/0	NA	1/2	NA			0/30	0	12.0
		2	0/0	NA	0/0	NA	No Trapping		0/1	NA	
		3	0/3	NA	8/29	28			2/27	7.4	
2013	Spring	1	No Trapping		0/0	NA	No Trapping		0/0	NA	0.0
		2			0/0	NA			0/0	NA	
		3			0/0	NA			0/0	NA	
	Summer	1	No Trapping		0/2	NA	No Trapping		No Trapping		0.0
		2			0/0	NA					
		3			0/0	NA					
	Autumn	1	No Trapping		0/0	NA	No Trapping		0/1	NA	3.6
		2			0/1	NA			0/1	NA	
		3			1/21	4.8			0/4	NA	
Total			28/384	7.3	42/301	14	2/45	4.4	44/574	7.7	10.4

Site-specific common vole abundance estimates ranged from 0 to 46 individuals/100TN. Large variation between the three replicate sites of each area was observed (Table 1, Figure 2). The highest average common vole abundance was 20 individuals/100TN observed in Weissach during summer 2011 (Figure 2).

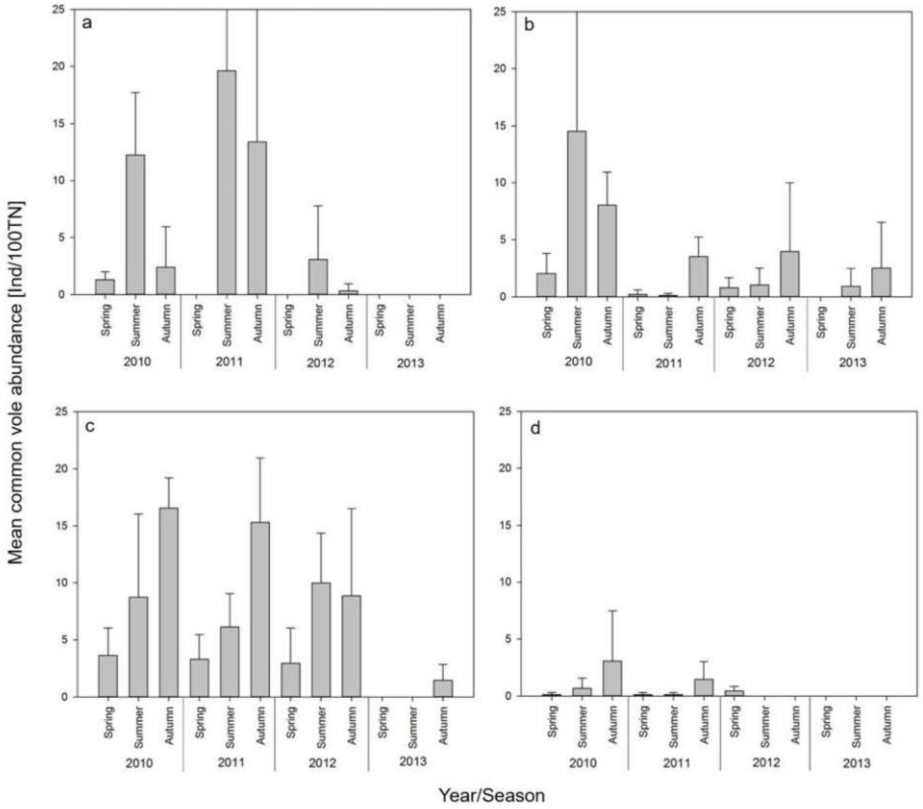

Figure 2. Population dynamics of common voles from 2010 to 2013 in four areas in Germany ((**a**): Weissach; (**b**): Jeeser; (**c**): Gotha; (**d**): Billerbeck)). Estimated mean abundance indices ± standard deviation as individuals per 100 trapping nights from three replicate grassland sites per area are based on live and snap trapping (see Table S1).

There were significant differences in abundances among study areas (ANOVA: F = 5.83, $p < 0.001$). More precisely, abundances of common voles were significantly lower in Billerbeck than in Gotha and in Weissach (Tukey's HSD: $p < 0.001$ and $p = 0.027$, respectively). A further statistical difference was found among seasons (F = 6.97, $p = 0.001$). Abundances were significantly lower in spring than in summer and autumn (Tukey's HSD: $p = 0.005$, respectively). There was also a difference among years (F = 2.91, $p = 0.038$) with abundances in 2010 tending to be higher than in 2013 (Tukey's HSD: $p = 0.064$).

Cytochrome *b* sequence analysis of 3–4 common voles from each trapping site confirmed the exclusive presence of the Central evolutionary lineage (Figure S1; for accession numbers see Table S3).

3.2. TULV Seroprevalence

Overall, 9% (119) of 1304 common voles had TULV-reactive antibodies. Most seropositive individuals were found in Jeeser (14%), Gotha (7.7%) and in Weissach (7.3%) while in Billerbeck, only two individuals were seropositive (Table 1).

The mean seroprevalence per site ranged between 0% and 28.0% with the highest prevalence found in Jeeser in autumn 2012. Statistically, mean seroprevalence over the

study period did not vary among study areas (ANOVA: F = 1.80, $p > 0.05$), seasons (F = 0.22, $p > 0.05$) or years (F = 1.02, $p > 0.05$). In a few cases, seroprevalence decreased from spring to summer and from summer to autumn. This could be observed in 2010 in Weissach and in 2012 in Gotha. In Jeeser, TULV-reactive antibodies were predominantly found in autumn. TULV-reactive antibodies were also detected in field voles, collected in Weissach, Jeeser and Gotha (Table S2).

Female common voles were more frequently captured than males (male:female = 1:1.2). There was an overall difference between sexes, with females being significantly less frequently seropositive compared to males (χ^2 =4.73, $p = 0.03$).

3.3. Relationship of TULV Seroprevalence with Common Vole Abundance

Due to low sample sizes in Billerbeck, this area was excluded from further analysis regarding TULV seroprevalence in common voles. Linear mixed modelling revealed varying impact of direct or delayed abundance on TULV seroprevalence (Table 2). There was an overall effect of abundance on TULV prevalence, which differed for direct and delayed dependence on abundance (Table 2). The abundance in the current season was negatively associated with TULV prevalence. Analysis of deviance on multi-level categorical factors (Wald chi-square tests) revealed that, overall, the study area was not a significant factor ($\chi^2 = 1.91$; $p = 0.39$), while in interaction with vole abundance, it had an overall significant effect ($\chi^2 = 9.01$; $p = 0.01$). The second model revealed a positive effect of vole abundance in the previous season on the subsequent prevalence. Despite the significance of the main factor, the interaction of delayed abundance and study area was not significant ($\chi^2 = 2.05$; $p = 0.36$) as well as the effect of study area alone ($\chi^2 = 2.93$; $p = 0.23$). The impact of direct dependence on abundance varied spatially with Weissach showing a negative association, Jeeser a positive and Gotha showing no direct dependence on abundance (Figure 3a). For delayed abundance dependency of seroprevalence, no geographical pattern emerged (Figure 3b).

Table 2. Direct and delayed effects of common vole abundance (as index) in interaction with study area (SA) on TULV seroprevalence in the host population. The categorical factor contained three levels with Weissach as the reference category. Number of observations each = 43, degrees of freedom each = 6. Bold values indicate significance of p value ($p < 0.05$). SE = standard error; SD = standard deviation; z = Wald statistics defined as Estimate/SE.

	Same Season (Direct Effect)				**Previous Season (Delayed Effect)**			
Parameter	**Estimate**	**SE**	**z**	***p***	**Estimate**	**SE**	**z**	***p***
Intercept	−1.735	0.333	−5.215	0	−2.675	0.344	−7.785	0
Abundance	**−0.028**	**0.012**	**−2.335**	**0.02**	**0.023**	**0.01**	**2.315**	**0.021**
Jeeser	−0.481	0.45	−1.069	0.285	0.755	0.454	1.662	0.097
Gotha	−0.643	0.5	−1.286	0.198	0.261	0.49	0.531	0.595
Abundance: Jeeser	**0.055**	**0.018**	**3.001**	**0.003**	**−0.024**	**0.02**	**−1.212**	**0.226**
Abundance: Gotha	0.022	0.03	0.73	0.466	−0.033	0.036	−0.926	0.354
Random factor	Variance	SD			Variance	SD		
Site:SA	0	0			0.129	0.359		
SA	0	0			0	0		

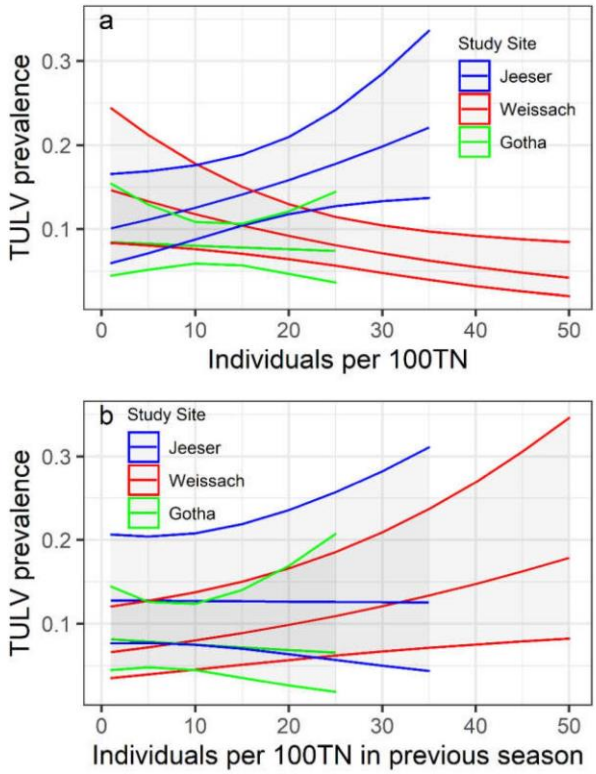

Figure 3. (**a**) Direct and (**b**) delayed effects of common vole abundance (as index with individuals per 100 trap nights) per study area on TULV seroprevalence in the host population.

3.4. Detection of TULV RNA and Sequence Analysis

RT-PCR investigations were performed for lung samples from common and field voles originating from snap trapping and from voles found dead in live traps. Initially, lung samples from 333 common voles and 100 field voles from all four trapping areas were analyzed for TULV S segment-specific RNA (Table S2, Table S3). Common voles from all four trapping areas tested positive for TULV RNA. The mean RNA prevalence ranged between 7.3% and 27.4% (Table S4). TULV RNA was detected in common voles trapped during three consecutive years (2010–2012) in Jeeser, Gotha and Weissach. TULV RNA was only detected in seropositive field voles from Gotha (Table S1). In one field vole from Weissach a PUUV RNA sequence was detected, indicating a spillover infection [56].

Phylogenetic analysis of the S segment sequences revealed a typical clustering with similar sequences from geographically close trapping sites (Figure S2a). In addition, as recently defined [17], sequences from Jeeser and Gotha clustered within the Central North (CEN.N) clade and showed characteristic in-frame insertions of 3 nt (CAA; glutamine codon) in all obtained S segment sequences at position 790 (counting according to TULV S segment, accession number NC_005227). This finding was accompanied by a high pairwise sequence identity among representatives of the same clade (Table S5). TULV S segment sequences from Billerbeck and Weissach were members of the Central South (CEN.S) clade (Figure S2a; Table S5). In the Moravia prototype isolate (classified as EST.S; [17]) and sequences from Billerbeck and Weissach, the 3 nt insertion was missing. Analyses of partial

L segment sequences showed the same patterns with sequences from Jeeser and Gotha in CEN.N clade, and sequences from Billerbeck and Weissach in the CEN.S clade (Figure S2b; see also Table S5). The M segment-based tree also showed the sequences from Jeeser and Gotha in CEN.N and sequences from Weissach in CEN.S; however, sequences from Billerbeck clustered here in the CEN.N clade (Figure S2c; see also Table S5).

Sequence variation in TULV S segment followed a strong isolation-by-distance relationship across all studied areas in Germany ($r^2 = 0.619$; Mantel test $p < 0.0001$; Figure 4). Consistent with larger geographic patterns of TULV variation [17], comparisons between study regions harboring different phylogenetic clades (TULV-CEN.S in the areas of Weissach and Billerbeck; TULV-CEN.N in the areas of Jeeser and Gotha) showed larger genetic divergence (*p*-distance: 18%–22%) than comparisons within TULV clades (*p*-distance: <13% between study areas). At the local scale, analysis revealed a highly significant isolation-by-distance pattern when all areas were tested jointly ($r^2 = 0.069$; Mantel test $p < 0.0001$; Figure 5). Separate Mantel tests according to study area demonstrated that this was largely driven by data from Weissach with up to four kilometers distance between sampling sites ($r^2 = 0.576$; $p < 0.0001$). Sequences from the other study areas with shorter maximum distances among sampling sites showed no significant isolation-by-distance patterns (Jeeser: $r^2 = 0.001$; $p = 0.274$; Gotha: $r^2 = 0.005$; $p = 0.512$; Billerbeck: $n = 2$ sequences, insufficient for statistical testing).

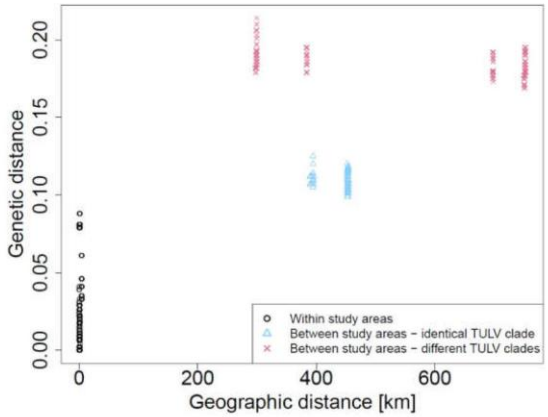

Figure 4. Isolation-by-distance relationship among TULV S segment sequences across the study areas in Germany. Red crosses represent data points for pairwise comparisons among the major phylogeographic clades TULV-CEN.S circulating in the study areas of Weissach and Billerbeck and TULV-CEN.N present in the study areas of Jeeser and Gotha.

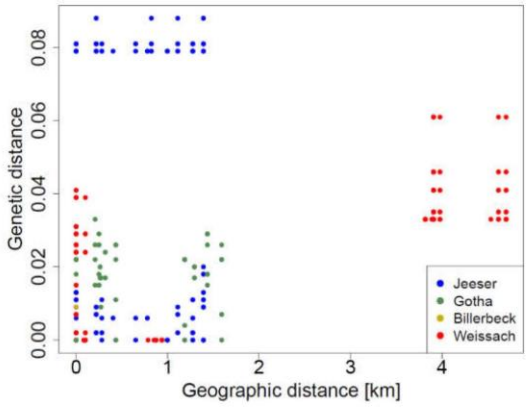

Figure 5. Relationships between TULV S segment sequences within the four study areas in Germany. Mantel tests detected significant isolation-by-distance patterns in the Weissach study area (red points; $p < 0.0001$) while there were no significant associations in the other sampling regions (all $p > 0.2$).

4. Discussion

The present study provides the first in depth account on spatial and temporal dynamics of TULV in relation to common vole population dynamics and their potential implications for molecular evolution in Central Europe. Conducting a multiannual monitoring field survey, which covered seasonal, annual and multi-annual fluctuations of common vole populations in four different regions, we were able to identify basal patterns of TULV dynamics within the rodent host populations in Germany. In comparison to PUUV, which was analyzed parallel to TULV in the same field survey (on additional forest plots; for details see [35,56]), TULV had a much broader geographical distribution (serological and RT-PCR detection in all four regions) and could be detected throughout Germany [9,57].

The estimated mean common vole abundance predominantly showed the typical seasonal fluctuations with lower numbers in spring, an increase during summer and a population peak in autumn (Figure 2; [26]). However, a few exceptions occurred in Weissach, in summer 2010 in Billerbeck and in summer 2012 in Gotha. Here, mean abundance peaked in summer. This deviation from the common seasonal pattern with autumn peaks could be due to small-scale processes. Common vole population dynamics are known to be influenced by various parameters such as predators and habitat factors [29] but also weather conditions [27,28]. At the small scale, dispersal capabilities of the common vole in relation to available nearby habitats can determine the local metapopulation structure [58]. These underlying, highly dynamic fluctuations may impact subsequent TULV dynamics at multiple scales.

The mean seroprevalences in common voles ranged in our study between 4.4% and 14.0%, with seasonal site-specific values between 0% and 28.0%. The range of the mean seroprevalences was similar to that observed in other studies in Germany (7.3%, [9], 16%, [6]), Austria (13.3%, [59]), France (7%, [60]), the Czech Republic (10%, [61], 9.7% [62]), Slovakia (6.6%, [63]), Belgium (7.7%, [64]) and Kazakhstan (15.6%, [65]). The mean RT-PCR detection rate in our study was at a similar level as the seroprevalences: it ranged between 7.3% and 27.4%, with seasonal site-specific values ranging between 0% and 37.5%. Results of previous studies revealed mean RNA detection rates of 15.6% [9] and 13.8% [57] in Germany and of 13.3% in Austria [59]. Similarly, a real-time RT-PCR-based study in the Netherlands indicated a TULV prevalence in the southern region of 41%, but of 12% to 45% in the northern regions [66]. The seasonal TULV RNA detection rate in another study in

Central Germany reached 58.3% at one site in spring [57]. In contrast to this, TULV was detected only rarely in field voles, confirming again the major role of the common vole as the reservoir of TULV, and that field voles are mostly affected by spillover infections [9].

Sex was a determining factor for TULV dynamics on an individual level because males had an overall higher likelihood to be TULV seropositive. This is consistent with previous work on TULV [60] and can in part be explained by larger male home ranges and longer dispersal distances [58,67] increasing the chances of intraspecific contacts, and potentially leading to seroconversion.

In contrast to our initial hypothesis, population-level TULV dynamics were not positively correlated to the current abundance. Our results suggest an overall positive delayed density dependence coupled with an overall negative direct density dependence (Table 2). This overall effect does appear to vary at lower spatial scales (interaction between abundance and site, Table 2). The generality of the assumption that high prevalence is always associated with high host abundance has been questioned repeatedly. Reil et al. [35], for example, found a strong seasonality in positive direct density dependence of PUUV. The latest results on PUUV in bank voles in Finland suggest that transient maternally derived immunity is a key feature of missing density dependence in populations [68]. For Sin nombre orthohantavirus (SNV) and its associated host, the deer mouse (*Peromyscus maniculatus*), a similar density dependence structure to the one presented here was described. Luis et al. [69] identified a strong delayed effect of deer mouse density on the prevalence of SNV. This is attributed to population fluctuations where the virus frequently becomes locally extinct due to missing host individuals. In such nonequilibrium, transient dynamics, peak host densities might not directly correspond to peak prevalence, as the virus survives at the metapopulation level rather than at a site-specific level. In these situations, immigration of nearby infected individuals is required, generating a time lag between the increase in host density and virus transmission at a particular site. Our data suggest that low winter survival in common vole populations with subsequent low spring abundances (Figure 2) could present such a bottleneck for site-specific TULV persistence. In this case, TULV might completely disappear from a plot and would need to be newly introduced by immigrating common voles from adjacent sources during the repopulation process [70,71]. Thereafter, it might take some time for the virus to spread within a newly established host population and, hence, the increase of TULV seroprevalence might be delayed in the following season. Given that the modern agricultural landscape supports a mosaic of suitable habitats for common voles, the degree of density dependence as well as the time lag is likely to vary between individual field sites depending on the distance to the nearest refuge as a source for recolonization to occur [72]. At a larger scale, this can be confirmed for TULV, as the different study areas varied in their expression of density-dependent patterns, likely reflecting differences in the landscape suitability structure and vole dynamics (Table 2). These results highlight that land-use patterns at the local and regional scale can have a large impact on the underlying pathogen dynamics and molecular evolution. Future work should therefore consider aspects of land use as explanatory variables for pathogen dynamics. However, our study had several limitations. Trapping could not be performed continuously at all sites in the last year of the study and the trap success and resulting lack of available sequences from the Billerbeck site might affect the large-scale applicability of the results. As this particular site was also characterized by a high prevalence of PUUV [56], the lack of samples limited the ability to investigate potential reassortment, though earlier publications using full genomes of TULV (and PUUV) or sequences from all genome segments have not provided evidence that reassortment is a common, or at least reasonably frequent, phenomenon in Central European phylogenetic clades and populations of these two orthohantavirus species [17,18,37,50].

Although we did not measure dispersal in the vole hosts directly, our molecular surveys conducted here indicate the buildup of isolation-by-distance patterns at the local scale, with sites closer together showing higher TULV relatedness compared to sites further apart. This can be interpreted as a host dispersal-driven metapopulation structure, where

TULV is more likely to be shared between sites closer together. At larger geographical scales between study areas, genetic distances between TULV continue to increase (see also [50]). Isolation-by-distance relationships are not detectable for comparisons between sequences belonging to different TULV clades (Figure 4), which is consistent with long-term evolutionary divergence into functionally different "genotypes" within TULV in Germany [17,18]. The phylogenetic analyses of the partial S and L segment sequences from all four trapping sites confirmed the expected classification to the CEN.N clade (Jeeser) and CEN.S clade (Billerbeck). This classification is also indicated by an in-frame insertion/deletion of a glutamine codon sequence in the S segment. Surprisingly, the partial M segment sequences from Billerbeck clustered within the CEN.N clade. Sequence evolution in this part of the genome might be governed more strongly by the function of the glycoproteins encoded by the M segment and related differences in the selection pressure compared to the other segments [17,73]. It remains to be tested with larger datasets if a reassortment event in the evolutionary history of the Billerbeck TULV strains further contributed to the phylogenetic patterns. Reassortment events have been detected by in vitro studies of other hantaviruses resulting in the exchange of the M segment but leaving the S and L segments unaltered [74,75]. Reassortment events were also discussed as the reason for the evolution of different hantaviruses in nature (for review see [76]).

5. Conclusions

This study focused on the temporal and spatial dynamics of multiannual common vole populations and highlighted determining factors. At the individual level, TULV infection risk was higher for males compared to females, likely reflecting different home ranges or aggressive interactions during the reproductive period. In contrast to our original hypothesis, TULV prevalence was negatively associated with current vole abundance, but positively dependent on the vole abundance of the previous season. This density dependence structure can be associated with transient, nonequilibrium host-pathogen dynamics, where frequent localized extinction events of hosts and pathogens (often during winter) on managed grasslands are followed by recolonization from nearby refuge areas. This observation is supported by isolation-by-distance patterns consistent with a dispersal-driven metapopulation structure at the local scale. However, the results are not consistent across all study sites, potentially reflecting different landscape structures mitigating the above-mentioned underlying mechanisms that lead to bottlenecks in local common vole populations.

Supplementary Materials: The following Supplementary Materials are available online at https://www.mdpi.com/article/10.3390/v13061132/s1, Figure S1: Phylogenetic tree of partial cytochrome *b* sequences of common voles from this study with reference sequences of the evolutionary lineages "Central", "Eastern", "Western" and "Italian", and field vole (*Microtus agrestis*) and bank vole (*Myodes glareolus*) sequences as outgroup, Figure S2: Phylogenetic trees of partial S (a), L (b) and M (c) segment sequences of Tula orthohantavirus (TULV), Table S1: Number of trapped common voles per year, season and trapping methodology as well as derived abundance index as individuals (Ind.) per 100 trap nights (TN), Table S2: Results of TULV-IgG ELISA and RT-PCR investigations of field voles, Table S3: Accession numbers of cytochrome *b* gene sequences of common voles from the four regions in Germany, Table S4: Results of RT-PCR investigations of common voles, Table S5: Pairwise sequence similarities of TULV S, M and L segment sequences from the four trapping sites and of reference sequences of clades CEN.N and CEN.S, Table S6: Accession numbers of all common vole-derived Tula orthohantavirus (TULV) sequences used for consensus tree reconstruction (identical sequences are indicated).

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and J.J.; Project Administration, J.J. and R.G.U.; Funding Acquisition, J.J. and R.G.U. All authors have read and agreed to the published version of the manuscript.

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(III) MULTIPLE INFECTIONS OF RODENTS WITH ZOONTOC PATHOGENS IN AUSTRIA

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ORIGINAL ARTICLES

Multiple Infections of Rodents with Zoonotic Pathogens in Austria

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Abstract

Rodents are important reservoirs for a large number of zoonotic pathogens. We examined the occurrence of 11 viral, bacterial, and parasitic agents in rodent populations in Austria, including three different hantaviruses, lymphocytic choriomeningitis virus, orthopox virus, *Leptospira* spp., *Borrelia* spp., *Rickettsia* spp., *Bartonella* spp., *Coxiella burnetii*, and *Toxoplasma gondii*. In 2008, 110 rodents of four species (40 *Clethrionomys glareolus*, 29 *Apodemus flavicollis*, 26 *Apodemus sylvaticus*, and 15 *Microtus arvalis*) were trapped at two rural sites in Lower Austria. Chest cavity fluid and samples of lung, spleen, kidney, liver, brain, and ear pinna skin were collected. We screened selected tissue samples for hantaviruses, lymphocytic choriomeningitis virus, orthopox viruses, *Leptospira*, *Borrelia*, *Rickettsia*, *Bartonella* spp., *C. burnetii*, and *T. gondii* by RT-PCR/PCR and detected nucleic acids of Tula hantavirus, *Leptospira* spp., *Borrelia afzelii*, *Rickettsia* spp., and different *Bartonella* species. Serological investigations were performed for hantaviruses, lymphocytic choriomeningitis virus, orthopox viruses, and *Rickettsia* spp. Here, Dobrava-Belgrade hantavirus-, Tula hantavirus-, lymphocytic choriomeningitis virus-, orthopox virus-, and rickettsia-specific antibodies were demonstrated. Puumala hantavirus, C. *burnetii*, and *T. gondii* were neither detected by RT-PCR/PCR nor by serological methods. In addition, multiple infections with up to three pathogens were shown in nine animals of three rodent species from different trapping sites. In conclusion, these results show that rodents in Austria may host multiple zoonotic pathogens. Our observation raises important questions regarding the interactions of different pathogens in the host, the countermeasures of the host's immune system, the impact of the host–pathogen interaction on the fitness of the host, and the spread of infectious agents among wild rodents and from those to other animals or humans.

Key Words: Rodents—Rodent-borne pathogens—Tick-borne pathogens—Austria—Multiple infections.

Introduction

In the last decades, the incidence of human diseases caused by zoonotic viruses, bacteria, and parasites that are associated with small mammal reservoirs appears to have increased (Meerburg et al. 2009). In Europe bank vole-associated Puumala virus (PUUV), different genotypes of Dobrava-Belgrade virus (DOBV) hosted by various *Apodemus* species and perhaps Tula virus (TULV) cause hemorrhagic fever with renal syndrome (HFRS) of different severity

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(Heyman et al. 2011, Klempa et al. 2013). For some other viral agents, such as lymphocytic choriomeningitis virus (LCMV) and cowpox virus (CPXV), a member of the genus *Orthopoxvirus* (OPV), the role of rodent reservoirs in Central Europe is unknown. LCMV causes infections in humans of varying severity from asymptomatic disease to severe meningitis (Emonet et al. 2007, Ceianu et al. 2008, Pérez-Ruiz et al. 2012), and sporadic CPXV infections have been described in humans, domestic, and zoo animals (Essbauer et al. 2010).

For bacterially induced zoonoses, leptospirosis is an emerging disease of global importance with a variation in the severity of symptoms (Bharti et al. 2003). Outbreaks are often associated with agricultural work or leisure activities involving exposure to freshwater (Desai et al. 2009). *Bartonella henselae* is the most important pathogenic *Bartonella* species in Europe. It is transmitted by cats and causes cat scratch disease and more rarely endocarditis, bacillary angiomatosis, and peliosis hepatis in immunodeficient patients (Kaiser et al. 2011). For many *Bartonella* spp., the pathogenicity is not known (*e.g.*, *B. taylorii, B. doshiae, B. birtlesii*), but some have been proven to cause endocarditis, bacterimia, and neuroretinitis (*B. grahamii*, *B. tamiae*) (Breitschwerdt et al. 2013). *Coxiella burnetii* may cause severe infections, *i.e.*, Q fever with pneumonia as typical symptom. The main sources for these infections are infected ruminants in which the agent may cause abortion and infertility. But other mammals, including rodents, are susceptible to infection with *C. burnetii* and may contribute to its transmission (Meerburg and Reusken 2011).

Rodents are also considered important reservoirs for different arthropod-borne bacteria (Hoogstraal 1967, Stanek and Strle 2003). *Borrelia afzelii*, the most prevalent spirochete causing Lyme disease in Europe, is perpetuated in a cycle involving rodents and *Ixodes ricinus* ticks (Richter et al. 2004a). *Borrelia bavariensis, B. spielmanii*, and *B. burgdorferi* sensu stricto (s.s.) are also associated with rodents, but generally infect fewer questing ticks (Richter et al. 2004b, Margos et al. 2009). Rickettsiosis is an increasing health problem in Europe (Parola and Raoult 2001), but studies in rodents as reservoirs are rare (Spitalská et al. 2008). Recently, a rodent survey identified *Rickettsia felis* and *R. helvetica* in rodents in southeastern Germany (Schex et al. 2011).

Rodents are also involved in the transmission cycles of endoparasites, such as *Toxoplasma gondii* (Mills and Childs 1998). Ingestion of *T. gondii*–infected tissues by felids, *e.g.*, domestic cats, may result in shedding of high numbers of environmentally resistant oocysts, from which infection is passed orally to humans (Dubey et al. 2004). Prenatal infections may cause abortion, and postnatal infections of immune-suppressed persons cause serious and occasionally fatal illness.

Here, we describe a survey for selected viruses, bacteria, and parasites in rodents captured in two areas in Lower Austria.

Materials and Methods

Rodent trapping and necropsy

In October, 2008, rodents were trapped in 565 snap traps during one night at five rural sites in the municipality of Laa an der Thaya and two rural sites in the municipality of Altenburg, northern Lower Austria, near the Czech border (Table 1, Fig. 1). Rodent necropsy and collection of chest cavity fluid (CCF) and tissue samples followed previously established standard protocols. Morphological species determination was confirmed by PCR and sequencing of the partial mitochondrial *cytochrome b* (*cyt b*) gene (Fink et al. 2010, Schlegel, et al. 2012b). Rodent species and genetic affiliations within species were determined by sequence comparisons against GenBank entries using the BLAST algorithm (www.ncbi.nlm.nih.gov) and against species-specific *cyt b* datasets covering all genetic lineages within these rodents (Michaux et al. 2003, Heckel et al. 2005, Michaux et al. 2005, Dubey et al. 2009, Wójcik et al. 2010, Sutter et al. 2013).

Serology

Serological investigations of CCF samples were performed using previously published protocols (Table 2).

Nucleic acid isolation

DNA and RNA were extracted from tissue samples using commercial kits (Qiagen Tissue Kit, QIAamp DNA Mini Kit, Qiagen, Hilden, Germany; Nucleospin DNA Tissue Kit, Macherey-Nagel, Düren, Germany; RTP DNA/RNA Virus Mini Kit, Invitek, Berlin, Germany) according to the manufacturers' instructions. Alternatively, RNA extraction was performed using a modified QIAzol extraction protocol (Schlegel et al. 2012a).

RT-PCR, PCR, and sequence analysis

Various published real-time and conventional RT-PCR/PCR and standard sequencing protocols were used for screening for viral, bacterial, and parasite-derived nucleic acids (Table 3). In addition, a conventional *Toxoplasma*-specific PCR and a novel *Bartonella*-specific real-time PCR targeting a fragment of the β-subunit of bacterial RNA polymerase were performed (for details, see Table 3).

Results

Rodent trapping

A total of 110 rodents were captured including 29 *Apodemus flavicollis*, 26 *A. sylvaticus*, 40 *Clethrionomys glareolus* (for the valid generic name of the bank vole, see Tesakov et al. 2010), and 15 *Microtus arvalis* (Table 1). The capture of 19.5 rodents consisting of only four species per 100 trap nights indicates a very high abundance of relatively low rodent diversity. According to the *cyt b* sequences, all rodents belonged to a single genetic lineage per species, each with large geographic distribution. Bank voles belonged to the Carpathian lineage (distribution, Eastern Europe/Balkans; Wojcik et al. 2010) and all common voles to the Eastern lineage (Eastern Europe; Heckel et al. 2005). Yellow-necked field mice and wood mice were represented by the clade C (Western Palaearctic distribution; Michaux et al. 2005) and the subclade 2b (Western/Central/Northern Europe; Michaux et al. 2003), respectively.

Detection of viral infections

Serological screening of bank voles for hantavirus (PUUV)-specific antibodies and PUUV/TULV S-specific RT-PCR revealed no positive animal (Table 1). One of the 29 (3.4%) yellow-necked field mice was seropositive in the DOBV-immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) (Table 1), whereas none of the wood

Table 1. Description of Trapping Sites, Rodents Trapped, and Pathogens Found

Trapping locality	*Site*	*Vegetation*	*Species*	*No. of trapped animals*	*Pathogen detection (no. of positive animals/total no. of animals analyzed)*	
					PCR or RT-PCR	*Serology*
Altenburg[a]	A1	Tall forbs, thick grass layer, bordering to a field	*Apodemus flavicollis*	4	*Leptospira* spp. (3/4), *Borrelia afzelii* (1/4)	*Rickettsia* spp. (1/4)
			Apodemus sylvaticus	1	None	*Rickettsia* spp. (1/1)
	A2	Bank slope with a thick grass and herbal layer	*Apodemus flavicollis*	1	None	None
			Apodemus sylvaticus	3	*Leptospira* spp. (2/3)	None
Laa an der Thaya[b]	L1	Overgrown sand pit with grassy ground, tall forbs, elder, and robinia bushery	*Apodemus flavicollis*	2	None	None
			Apodemus sylvaticus	4	*Borrelia afzelii* (1/4)	None
			Clethrionomys glareolus	8	*Borrelia afzelii* (1/8)	*Rickettsia* spp. (1/8)
			Microtus arvalis	7	TULV (1/7), *Leptospira* spp. (1/7), *Borrelia afzelii* (4/7), *Rickettsia* spp.(2/7), *Bartonella taylorii* (2/7)	TULV (1/7), *Rickettsia* spp. (1/7)
	L2	Edge of a robinia–ash forest, bordering to a field	*Apodemus flavicollis*	10	*Borrelia afzelii* (1/10), *Bartonella taylorii* (1/10)	DOBV (1/10)
			Apodemus sylvaticus	5	*Bartonella taylorii* (1/5), *Bartonella grahamii* (2/5)	LCMV (1/5)[c]
			Clethrionomys glareolus	5	None	*Rickettsia* spp. (1/5)
			Microtus arvalis	4	TULV (1/4), *Borrelia afzelii* (2/4)	TULV (1/4), *Rickettsia* spp. (1/4)
	L3	Base of an embankment at the Thaya with a thick hedge of robinia, blackthorn, ash, and *Euonymus europaeus*	*Apodemus flavicollis*	4	None	*Rickettsia* spp. (1/4)
			Apodemus sylvaticus	8	*Bartonella birtlesii* (1/8), *Bartonella taylorii* (2/8)	None
			Clethrionomys glareolus	5	*Leptospira* spp. (2/5), *Borrelia afzelii* (1/5)	None
			Microtus arvalis	4	*Borrelia afzelii* (2/4), *Borrelia garinii* (1/4)	*Rickettsia* spp. (1/4)
	L4	Robinia and ash forest with grassy ground and local stinging-nettle populations	*Apodemus flavicollis*	4	*Borrelia afzelii* (1/4), *Bartonella taylorii* (1/4)	None
			Clethrionomys glareolus	11	*Leptospira* spp. (1/10[d]), *Borrelia afzelii* (1/11), *Rickettsia* spp. (1/10[d])	*Rickettsia* spp. (1/11), OPV (1/7[d])[c]
	L5	Northern bank of the Thaya with extended goldenrod vegetation and moist ground	*Apodemus flavicollis*	4	*Bartonella taylorii* (1/3[d])	None
			Apodemus sylvaticus	5	*Bartonella taylorii* (1/5)	*Rickettsia* spp. (1/5)
			Clethrionomys glareolus	11	*Bartonella doshiae* (1/11)	*Rickettsia* spp. (1/11)

[a]A1: 65 traps at WGS 84: 48.63086 N 15.61078 E 281 m, and A2: 135 traps at WGS84: 48.62995 N 15.61493 E 266 m.
[b]L1: 65 traps at WGS 84: 48.73685 N 16.33875 E 182 m; L2: 70 traps at WGS 84: 48.73583 N 16.33818 E 184 m; L3: 100 traps at WGS 84: 48.73587 N 16.34259 E 183 m; L4: 50 traps at WGS 84: 48.73450 N 16.34060 E 178 m; L5: 80 traps at WGS 84: 48.73571 N 16.34543 E 182 m.
[c]The Pan-arenavirus RT-PCR and the OPV-PCR were negative.
[d]For this analysis samples were not available for all animals.
TULV, Tula virus; DOBV, Dobrava-Belgrade virus; OPV, orthopox virus; LCMV, lymphocytic choriomeningitis virus.

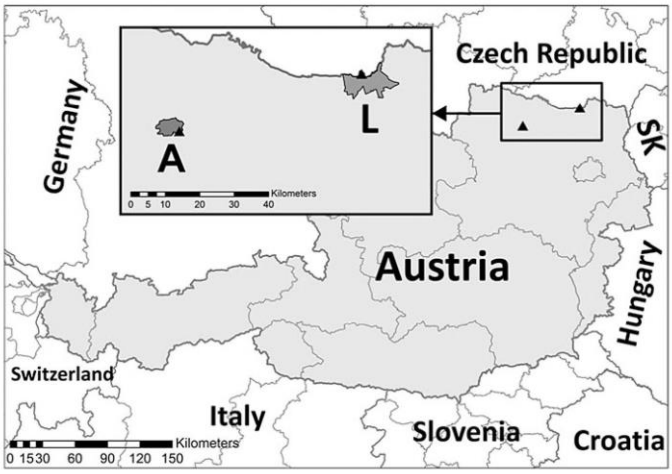

FIG. 1. Map of trapping sites at the municipality Laa an der Thaya (L) and the municipality Altenburg (A) in Lower Austria. SK, Slovakia.

mice contained DOBV-specific antibodies. Hantavirus RNA was not detected in any of the investigated *Apodemus*-derived lung samples. TULV was the only hantavirus detected by serological and molecular methods in two of the 15 (13.3%) common voles (Table 1). A phylogenetic analysis of the obtained S segment sequences (accession nos. KF184327 and KF184328) demonstrated their close relationship to previously published TULV sequences from Austria (similarity of 92–98%) and Slovakia and Czech Republic (94–97%; Bowen et al. 1997; data not shown). LCMV-specific antibodies were detected in one of 26 (3.8%) wood mice, but not in any other species. Subsequent Pan-arenavirus RT-PCR analysis did not amplify any LCMV-specific RNA (Table 1). One CCF sample of 29 analyzed bank voles produced a weak signal in the OPV-IFA, but OPV-DNA was not detected by PCR in any rodent (Table 1).

Detection of bacterial and T. gondii *infections*

For the bacteria, the *lipl32 Leptospira*-PCR assay revealed a specific product for eight of 109 (7.3%) examined kidney samples (Table 1). The duplex PCR identified *L. kirschneri* in two wood mice and one yellow-necked field mouse from Altenburg, whereas products indicating infection with the genomospecies *L. interrogans*, *L. borgpetersenii*, *L. weilii*, *L. noguchii*, *L. santarosai*, or *L. meyeri* were amplified from two bank voles and one common vole from Laa an der Thaya. Of the remaining two *lipl32*-PCR positive rodents, one was negative (bank vole) in the duplex PCR approach; the other (yellow-necked field mouse) could not be further analyzed by this assay.

Borrelia-specific DNA was detected by nested PCR in a total of 16 animals (14.8%) of all four examined species, with the highest prevalence (53.3%) in common voles (Table 1). Subsequent sequencing confirmed *B. afzelii*–specific DNA for 15 samples; for one common vole, a co-infection by *B. afzelii* and *B. garinii* was found (Table 4).

Indirect IFA investigation using *Rickettsia conorii* as the spotted-fever group (SFG) antigen demonstrated reactivity in 11 animals of all four species and both trapping sites, most frequently in *M. arvalis* ($n = 2/15$; 13.3%) (Table 1). Pan-rickettsial PCR analysis revealed three positive tissue samples (Table 1). The amplification of the *omp*B fragment

TABLE 2. OVERVIEW OF THE SEROLOGICAL METHODS USED FOR SCREENING RODENTS FOR ZOONOTIC AGENTS

Pathogen	*Method*	*Reference*
Puumala virus	ELISA	Mertens et al. 2011
Dobrava–Belgrade virus	ELISA	Schlegel et al. 2009
Tula virus	ELISA	Schlegel et al. 2012a
Lymphocytic choriomeningitis virus	Indirect Immunofluorescence	Ceianu et al. 2008, Coulybaly-N'Golo et al. 2011
Orthopox virus[a]	Indirect Immunofluorescence	Appl et al. 2013
Rickettsia spp.	Indirect Immunofluorescence	*Rickettsia conorii* Panbio IF Kit; for details see Schex et al. 2011

[a]Due to the cross-reactivity of orthopox viruses, this assay detects also cowpox virus–specific antibodies.
ELISA, enzyme-linked immunosorbent assay.

TABLE 3. OVERVIEW OF THE MOLECULAR METHODS USED FOR SCREENING RODENT SAMPLES FOR ZOONOTIC AGENTS

Pathogen	*Tissue*	*Method*	*Target*	*Reference*
Puumala virus	Lung	Conventional RT-PCR	Partial S segment (760 bp)	Essbauer et al. 2006
Dobrava–Belgrade virus	Lung	Conventional RT-PCR	Partial L segment	Klempa et al. 2006
Tula virus	Lung	Conventional RT-PCR and direct sequencing	Partial S segment (760 bp)	Essbauer et al. 2006
Lymphocytic choriomeningitis virus	Spleen	Conventional RT-PCR	Partial Lassavirus L gene	Coulybaly-N'Golo et al. 2011, Vieth et al. 2007
Orthopox virus (OPV)[a]	Liver	Real-time PCR	Partial hemagglutinin gene	Qiagen-Artus Orthopox LC PCR Kit; Olson et al. 2004
Leptospira spp.	Kidney	Conventional PCR	Partial *flaB* (563-bp fragment)	Gravekamp et al.1993
			Partial *secY* (285-bp fragment)	Bal et al. 1994, Levett et al. 2005
			Partial *lipl* (423-bp fragment)	Haake et al. 2000, Mayer-Scholl et al. 2011
Borrelia spp.	Skin	Nested conventional PCR and direct sequencing	Partial 16S rRNA (600 bp)	Richter et al. 2006, 2013
Rickettsia spp.	Skin	Screening real-time PCR	Partial *gltA*	Wölfel et al. 2006,
		Conventional PCR	Partial *ompB*	Schex et al. 2011
Bartonella spp.	Spleen	Real-time screening PCR	Partial *rpoB* (78 bp)	This paper[b]
		Conventional confirmatory PCR and direct sequencing	ITS (419–565 bp)	Maggi and Breitschwerdt 2005
Coxiella burnetii	Liver	Screening real-time PCR	Partial *IS1111*	Schrader et al. 2000
		Nested conventional PCR	Partial *com1*	Zhang et al. 1998
Toxoplasma gondii	Brain	Conventional PCR	529-bp repeat	Reischl et al. 2003, Homan et al. 2000, this paper[c]

[a]Detects OPV including also cowpox virus (CPXV).
[b]With QuantiFast Probe PCR kit (Qiagen) according to the manufacturers' protocol using primers BART F1(5′-AGA AGA GTT TGT TGT TTG CC), BART F2 (5′-AGA AGA GTT TGT TGT TTG TC), BART R (5′-GAA ACA TCC ATC AAA TCA ACA TG) and LNA probe BART-P (5′-FAM- AAA CTT CAC CAG CAT GA-BHQ1.
[c]Primers TOX-8 (0.5 μM) in combination with Tox5 (0.5 μM) were used with the Dynazyme II F-501L polymerase (Finzyme, Espoo, Finland). Cycling was performed at 94°C for 1 min, followed by 35 cycles of 60°C for 1 min, 72°C for 1 min, and 94°C for 1 min, and a final extension at 72°C for 10 min.
FAM, 6-carboxyfluorescein; BHQ1, black hole quencher 1.

was not possible, and thus the species could not be characterized.

Initial real-time PCR analysis for *Bartonella* produced a total of 21 positive samples, but only 12 samples were confirmed by conventional PCR (Table 1). Subsequent sequencing identified *B. taylorii* in three yellow-necked field mice, three wood mice, and two common voles. *B. grahamii* was exclusively found in two wood mice, *B. doshiae* in one bank vole, and *B. birtlesii* in one wood mouse.

TABLE 4. DETECTION OF MULTIPLE INFECTIONS IN AUSTRIAN RODENTS

Species (frequency of multiple infection)	*Trapping site*	*Pathogens*[a]
Apodemus flavicollis (2/29)	Altenburg, site 1	*Borrelia afzelii* and *Leptospira* spp.
	Laa an der Thaya, site 4	*Borrelia afzelii* and *Bartonella taylorii*
Clethrionomys glareolus (1/40)	Laa an der Thaya, site 1	*Borrelia afzelii* and *Rickettsia* spp. (serology)
Microtus arvalis (6/15)	Laa an der Thaya, site 1	*Borrelia afzelii*, *Rickettsia* spp. (PCR)
	Laa an der Thaya, site 1	*Borrelia afzelii*, *Bartonella taylorii* and *Rickettsia* spp. (serology)
	Laa an der Thaya, site 1	*Borrelia afzelii,* Tula virus (RT-PCR, serology), *Bartonella taylorii*
	Laa an der Thaya, site 2	*Borrelia afzelii* and *Rickettsia* spp. (serology)
	Laa an der Thaya, site 2	*Borrelia afzelii,* Tula virus (RT-PCR, serology)
	Laa an der Thaya, site 3	*Borrelia afzelii, Borrelia garinii*

[a]Detection method is given in brackets for the aforementioned pathogen, when RT-PCR/PCR and a serological method were used.

C. burnetii and *T. gondii* infections were not detected in any of the animals.

Multiple infections

Double and triple infections were detected in seven and two of 110 (6.4% and 1.8%) rodents respectively, comprising three of four rodent species (Table 4). Common voles were most frequently infected by more than one pathogen. *B. afzelii* was detected in all multiply infected animals. Three multiply infected animals harbored *B. taylorii*. Co-infections with *Rickettsia* spp. were demonstrated in three of four animals only by serology. Both common voles harboring TULV RNA also contained DNA of *B. afzelii* and one additionally DNA of *B. taylorii*.

Discussion

This molecular and serological survey of 110 rodents from Lower Austria demonstrated 50 animals being infected by at least one pathogen, including hantaviruses (TULV and DOBV), LCMV, OPV, *Leptospira* spp., *B. afzelii*, *Rickettsia* spp., and different *Bartonella* species. In line with these results, human infections with several of these pathogens have been reported in Austria, *i.e.*, CPXV, as an important OPV, *Leptospira* spp., *Borrelia* spp., and *Rickettsia* of the SFG group (Stanek et al. 2009, Glatz et al. 2010, Radl et al. 2011, Sonnleitner et al. 2012). Due to the lack of data, the impact on human health of LCMV, TULV, DOBV, *Bartonella* spp., and *B. grahamii* detected in rodents in this part of Austria requires increased awareness of the Austrian physicians.

PUUV was identified as causative agent in some patients from Austria, but no clinical cases have been reported for Lower Austria, although this virus was detected in bank voles in that area (Aberle et al. 1999, Plyusnina et al. 2006). The failure to detect PUUV in our sample of bank voles may indicate that this virus was absent at the investigated sites in 2008 or present at a very low prevalence, even though a relatively high number of human hantavirus cases was detected that year in Austria ($n = 33$; Heyman et al. 2011). The detection of TULV in common voles and their similarity to other Austrian TULV sequences confirmed the circulation of this hantavirus in Austria (Bowen et al. 1997). For further analysis on the phylogeography and molecular evolution of TULV, future investigations should target not only the S but also the M segment. Importantly, the potential pathogenicity of this hantavirus needs additional studies in human patients and risk groups (Mertens et al. 2011). To confirm the presence of DOBV in Austria, as indicated by our observation of DOBV-reactive antibodies in a yellow-necked field mouse, reservoir studies and a molecular identification of the DOBV genotype are required.

We have confirmed herein that wood mice from Austria are susceptible to LCMV or closely related arenaviruses, as has already been shown for wood mice from Spain (Ledesma et al. 2009). In contrast to previous investigations in Europe (Kallio-Kokko et al. 2006), we did not find hints for LCMV infection in yellow-necked field mice, bank voles, and common voles. The observed low OPV prevalence in rodents contrasts the high prevalences of OPV-reactive antibodies in different rodent species reported in previous studies for other parts of Central Europe (Essbauer et al. 2009, Kinnunen et al. 2011).

The proportion of *Leptospira*-positive rodents and the presence of several *Leptospira* species in different rodent species is in accordance with previous studies (Sebek et al.1989). The detection of four different *Bartonella* spp. in our study confirmed the presence of these bacteria in Central Europe (Telfer et al. 2007, Kaiser et al. 2011, Janecek et al. 2012). *B. taylorii* was the most frequently detected species without apparent host specificity. In contrast, we found *B. grahamii* only in wood mice, although it has been shown in many small sylvatic mammals (Holmberg et al. 2003). *B. doshiae* was detected solely in bank voles, supporting previous observations in Slovenia (Knap et al. 2007). In accordance with its first description in *Apodemus* spp. (Bermond et al. 2000), *B. birtlesii* was only found in wood mice.

Nearly 15% of our sampled rodents were infected by *B. afzelii.* Although specific rodent-associated genospecies may be better adapted to particular rodent species (Richter et al. 2004a,b, Richter et al. 2011), we observed no specificity in our samples. Presence of *Borrelia* DNA in the skin fails to prove reservoir status, but demonstrates contact with an infected tick. This might be the case for the common vole in which DNA of bird-associated *B. garinii* was detected. Information on the role of rodents in the natural cycle of different *Rickettsia* species is limited. Epidemiological data mostly based on questing ticks revealed the presence of several species of the SFG group in Austria (Blaschitz et al. 2008, Dobler et al. 2008). Detection of rickettsia DNA and rickettsia-specific antibodies in our study confirmed results previously reported for Bavarian rodents (Schex et al. 2011).

In contrast to reports of Q fever infection in humans (Kaplan and Bertagna 1955, Allenberger et al. 2009) and rodents in Tyrol (Stützner et al. 1979), we failed to detect C. *burnetii*. The occurrence of *C. burnetii* in rodents seems to be related to anthropogenic impact, such as farming of goats, cattle, and sheep (Webster et al. 1996, Reusken et al. 2011). In contrast, the agent was not detected in rodents inhabiting sylvatic sites (Reháček et al. 1993). The failure to detect *T. gondii* in rodent samples was not unexpected because a large study conducted in the Czech Republic examining rodents as potential intermediate hosts revealed a prevalence of only 0.9% viable *T. gondii* in 5166 small mammals of 17 species (Hejlíček and Literak 1998). Older rodents and rodents trapped close to dwellings are more likely to have seroconverted (Dabritz et al. 2008). Thus, in our study, the character of the trapping site and age of the trapped rodents may have influenced the likelihood to detect *C. burnetii* and *T. gondii* infection.

Information on multiple infections in rodents is sparse. In our study, we found seven of 110 (6.4%) of the animals infected by two pathogens and additionally two of 110 (1.8%) by three. In a study on 44 rodents in Croatia, dual infections with hantaviruses and *Leptospira* (16%), hantaviruses and *Babesia* (5%), and *Leptospira* and *Babesia* (2%), and triple infections in 7% of the rodents were demonstrated (Tadin et al. 2012). Moreover, interactions of pathogens, *i.e.*, of CPXV, *Babesia microti*, *Bartonella* spp., and *Anaplasma phagocytophilum*, have been identified in field voles (Telfer et al. 2010).

Conclusions

In summary, we demonstrate in our pilot study at two selected sample sites that multiple rodent-associated pathogens occur in Austria. Despite the relatively low number of

collected and tested animals, we detected several pathogens with zoonotic potential. Also, coinfections with more than one pathogen do not seem uncommon in wildlife. Thus, our results indicate that rodents may be able to transmit a multitude of pathogens directly or indirectly to other animals or humans. Future investigations will have to examine the potential interactions of different pathogens, their influence on the reservoir competence and fitness of the host, and the underlying molecular mechanisms, as well as the potential public health impact of these multiple infections. Further studies also have to examine whether and which site-specific, seasonal, and annual variations of the prevalence within reservoir and transmission risk occur.

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No competing financial interests exist for any of the authors.

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Publication I:

Schmidt S, Saxenhofer M, Drewes S, Schlegel M, Wanka KM, Frank R, Klimpel S, von Blanckenhagen F, Maaz D, Herden C, Freise J, Wolf R, Stubbe M, Borkenhagen P, Ansorge H, Eccard JA, Lang J, Jourdain E, Jacob J, Mariannaeu P, Heckel G, Ulrich RG (2016) High genetic structuring of Tula hantavirus. Arch Virol 161(5):1135-1149

Schmidt, S:	Rodent trapping Small mammal dissection Molecular species determination ELISA RT-PCR Sequencing Submitting sequence data to GenBank Writing manuscript Proofreading manuscript
Saxenhofer, M:	Phylogenetic analysis Writing manuscript Proofreading manuscript
Drewes, S:	Small mammal dissection ELISA RT-PCR Sequencing Phylogenetic analysis Proofreading manuscript
Schlegel, M:	Small mammal dissection ELISA RT-PCR Sequencing Proofreading manuscript
Wanka, KM:	Small mammal dissection ELISA
Frank, R:	small mammal dissection ELISA RT-PCR Sequencing
Klimpel, S:	Supervision
von Blanckenhagen, F:	Rodent trapping
Maaz, D:	Rodent trapping Small mammal dissection ELISA RT-PCR
Herden, C:	Rodent trapping

Freise, J:	Rodent trapping
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Stubbe, M:	Rodent trapping
Borkenhagen, P:	Rodent trapping
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Eccard, JA:	Rodent trapping
Lang, J:	Rodent trapping
Jourdain, E:	Rodent trapping
Jacob, J:	Rodent trapping Proofreading manuscript
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Ulrich, RG:	Study design Supervision Funding acquisition Writing manuscript Proofreading manuscript

Publication II:

Schmidt S, Reil D, Jeske K, Drewes S, Rosenfeld UM, Fischer S, Spieling NG, Labutin A, Heckel G, Jacob J, Ulrich RG, Imholt C (2021) Spatial and temporal dynamics and molecular evolution of Tula orthohantavirus in German vole populations. Viruses 13:1132

Schmidt, S:	Rodent trapping Small mammal dissection Molecular species determination Molecular sex determination ELISA RT-PCR Sequencing (S segment) Writing manuscript Proofreading manuscript
Reil, D:	Rodent trapping Small mammal dissection Abundance analysis Writing manuscript Proofreading manuscript
Jeske, K:	RT-PCR Sequencing (M and L segment) Writing manuscript Proofreading manuscript
Drewes, S:	Rodent trapping Small mammal dissection Proofreading manuscript
Rosenfeld, UM:	Rodent trapping Small mammal dissection
Fischer, S:	Rodent trapping Small mammal dissection Proofreading manuscript
Spierling, NG:	Rodent trapping Small mammal dissection
Labutin, A:	Phylogenetic analysis
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Publication III:

Schmidt S, Essbauer SS; Mayer-Scholl A, Poppert S, Schmidt-Chanasit J, Klempa B, Henning K, Schares G, Groschup MH, Spitzenberger F, Richter D, Heckel G, Ulrich RG (2014) Multiple infections of rodents with zoonotic pathogens in Austria. Vector Borne Zoonotic Dis 14(7):467-476

Schmidt, S:	molecular species determination analysis (orthohantavirus) organizing and collection of data of all studied pathogens alignment for phylogenetic analysis writing manuscript proofreading manuscript
Essbauer, SS:	analysis (orthopox virus, *Rickettsia* spp.) proofreading manuscript
Mayer-Scholl, A:	analysis (*Leptospira* spp.) proofreading manuscript
Poppert, S:	analysis (*Bartonella* spp.) proofreading manuscript
Schmidt-Chanasit, J:	analysis (LCMV)
Klempa, B:	supervision of hantavirus investigations
Henning, K:	analysis (*Coxiella burnetii*)
Schares, G:	analysis (*Toxoplasma gondii*)
Groschup, MH:	supervision
Spitzenberger, F:	rodent trapping proofreading manuscript
Richter, D:	analysis (*Borrelia* spp.) proofreading manuscript
Heckel, G:	phylogenetic analysis TULV proofreading manuscript
Ulrich, RG:	Study design Supervision Writing manuscript Proofreading manuscript

4. RESULTS AND DISCUSSION

4.1. Distribution, prevalence, and host association of TULV

4.1.1. Distribution and prevalence

To gain further insight in the distribution and host association of TULV in Central Europe, a total of 1361 rodents were captured at 87 sites from 2004-2013: 72 sites in 13 federal states in Germany, two sites in Luxembourg near the German border, seven sites in two municipalities in Austria and six sites in two administrative districts in France. Individuals representing six rodent species could be trapped: 911 common voles, 324 field voles, 31 water voles, 40 bank voles, 29 yellow-necked field mice and 26 wood or long-tailed field mice (*Apodemus sylvaticus*). Additional 1042 blood samples from common voles and 50 from field voles were obtained in a capture-mark-recapture study at four of these 72 trapping sites in Germany: Jeeser in Mecklenburg-Western Pomerania, North Germany, (II) Gotha in Thuringia, Central Germany, (III) Billerbeck in North-Rhine Westphalia, Western Germany and (IV) Weissach in Baden-Wuerttemberg, South Germany.

Analysis of TULV-reactive antibodies in blood (or chest cavity fluid) and TULV RNA in lung of common voles, field voles and water voles revealed positive animals at 53.6 % (confidence interval 95 % (CI 95): 42.3-64.5) of all trapping sites: One of two sites in Luxembourg, three of six sites in both administrative districts in France, 39 of 72 sites in Germany in all sampled federal states and at two out of three locations in Austria where common voles were trapped (Figure 2). All three tested species had detectable antibodies in their blood or viral RNA in their lung (Paper III, Table 1; Paper I, Table 1; Paper II, Table 1).

The overall prevalence in common voles was low. Seroprevalence ranged from 0 -19 % (mean (all sites): 8.5 %; mean (positive sites): 9.0 %), RNA could be detected in 0 – 37.5 % of captured common voles (mean (all sites): 15.3 %; mean (positive sites): 16.7 %). The results of these studies (Paper I, II and III) indicate that reverse transcription polymerase chain reaction (RT-PCR) analysis is the more reliable tool when investigating TULV infection in voles. 10.2 % of analyzed common voles have been

positive in RT-PCR only, whereas the detection of antibodies failed (Table 2). In part, this can be explained with the materials used. For animals found dead, no full blood samples could be obtained. Instead, the chest cavity had to be rinsed with phosphate-buffered saline (PBS) solution, resulting in a diluted sample. In our capture-mark-recapture study, seroprevalence was higher for the same trapping location using less diluted blood samples derived from live trapping compared to the more diluted chest cavity fluid that had to be used for snap trapped animals (e.g., 9.8 % vs. 2.5 % in common voles from Gotha and 15.4 % vs. 0 % for common voles from Jeeser). But since the seroprevalence from blood samples was still lower than the RNA prevalence from animals found dead in live traps from the very same site (9.8 % vs. 14.3 % for Gotha, 8.7 % vs. 11.5 % for Weissach and 15.4 % vs. 24 % for Jeeser) it also seems possible that common voles as the primary host do not mount a strong antibody response towards TULV.

TULV has been reported in *Microtus* spp. voles from many different European as well as Asian countries (Table 1). The reported prevalence in those studies was similar to that found in our studies, ranging from 5.6 % to 33.3 % in common voles. To this date, no indication of TULV infection was detected in Spain, where the common vole population is more isolated than other European populations (Jeske et al., 2021b).

This indicates that TULV is a very wide-spread and common infection in wild living voles, which was confirmed in this study not only on country-level but on a much finer scale throughout several federal states and municipalities. The number of trapping sites where TULV is present may even be higher than 53.6 % (CI 95: 42.3-64.5) reported here. Trapping success at TULV-positive locations was much higher than for TULV-negative. A mean of 2 common vole individuals could be trapped at sites where no indication of infection was found. Regarding the low prevalence, TULV presence cannot be excluded with certainty for those locations where trappings were less successful.

Table 1: Molecular evidence of TULV in Europe and Asia

Country	**Literature**
Austria	Bowen et al., 1998
Belgium	Heyman et al., 2002
China	Guo et al., 2019; Chen et al., 2019
Crimean Region	Yashina et al., 2015
Croatia	Scharninghausen et al., 2002; Tadin et al., 2016
Czech Republic	Plyusnin et al., 1995; Heroldová et al., 2010; Saxenhofer et al., 2019
France	Plyusnina et al., 2007; Deter et al., 2008
Germany	Klempa et al., 2003; Schmidt-Chanasit et al., 2010; Mertens et al., 2011a; Jeske et al., 2021a
Hungary	Jakab et al., 2008; Kurucz et al., 2018
Kazakhstan	Plyusnina et al., 2008b
Lithuania	Jeske et al., 2022
Netherlands	Reusken et al., 2008; Maas et al., 2017
Poland	Song et al., 2004
Russia	Plyusnin et al., 1994
Serbia	Song et al., 2002; Nikolic et al., 2014
Slovenia	Korva et al., 2009 and 2013b
Slovakia	Sibold et al., 1995 and 1999

4.1.2. Host association

TULV was first detected in 1994 in the common vole (Plyusnin et al., 1994) which is assumed to be the primary host. Since then, infection has been described in various other Arvicolinae rodents: field voles, sibling voles, narrow-headed voles, water voles and European pine voles (Song et al., 2002; Scharninghausen et al., 2002; Korva et al. 2009; Schmidt-Chanasit et al., 2010; Schlegel et al., 2012) as well as some Murinae rodents: pygmy or Ural field mouse (*Apodemus uralensis*) and wood mouse (Heroldová et al., 2010).

Spillover infections of hantaviruses have been described but seem to be a rare event in general (e.g., Binder et al., 2020a; Klingström et al., 2002). For non-reservoir rodent hosts a measurable immune response followed by virus clearance has been reported (e.g., Spengler et al., 2013; Schountz et al., 2014). Furthermore, *in vitro* experiments with bank vole- and common vole-derived cell lines have shown that PUUV does not replicate in common vole-derived cell lines and TULV not in bank vole-derived cell lines (Binder et al., 2019).

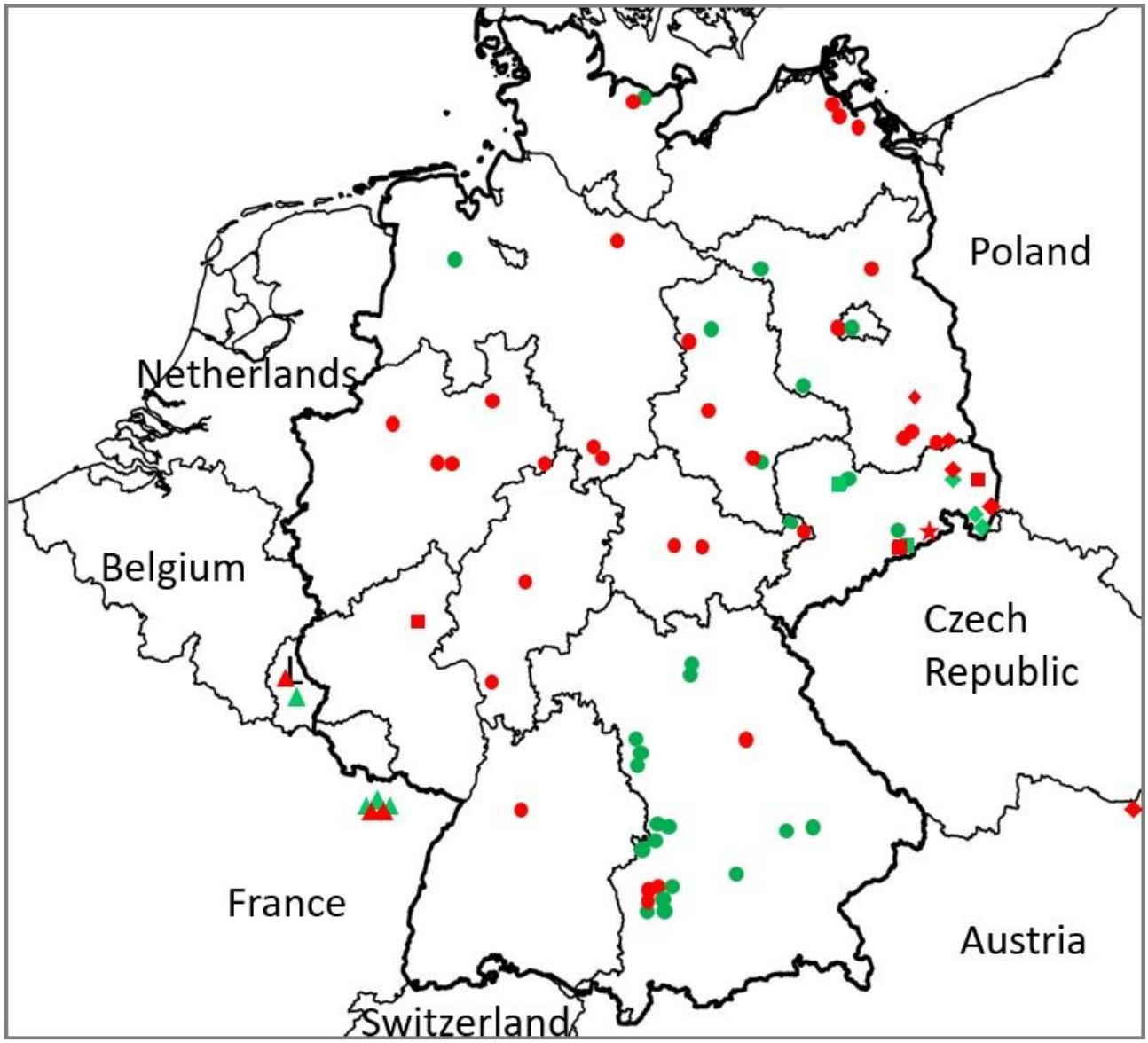

Figure 2: Presence of TULV-specific antibodies and/or RNA in all three analyzed vole species at different trapping sites in Germany, Luxembourg (L), Austria and France; Figure was first presented in Schmidt et al., 2016, modified
red = TULV-specific antibodies and/or RNA detected, green = no TULV antibodies and/or RNA detected
genetic lineage of the rodent host *Microtus arvalis:* dot: Central lineage, diamond: Eastern lineage, triangle: Western lineage, asterisk: trapping site with animals of different lineages (Eastern/Central), square: lineage not tested; mainly because no common voles were trapped

Still, the multitude of infected species raises questions about the host association of TULV. This virus has been detected in field voles and water voles without presence of its primary host at some locations (Schlegel et al., 2012, Schmidt-Chanasit et al., 2010), at other trapping sites the prevalence in field voles was higher than in common voles (27.6 % and 11.8 %, respectively) (Scharninghausen et al., 2002). It has been suggested that these infections are not an accidental spillover but that both species might represent an alternate host for TULV, maybe even equally suited for viral replication.

This work contradicts this hypothesis. Most TULV-positive voles were common voles: 16.3 % showed signs of past or ongoing infection with TULV compared to 8.1 % of field voles and 9.7 % of water voles (Table 2). TULV-positive common voles were sympatric with field voles at 11 trapping sites, only at 5 of them any sign of TULV infection could be detected in field voles. TULV-RNA could only be detected in water voles when they were sharing their habitat with RNA-positive common voles.

Table 2: Comparison of TULV detection in different vole species

		positive in IgG ELISA only	positive in RT-PCR only	positive in both	total number tested positive in at least one assay
common voles	Paper I	9/670 (1.3 %) CI 95 0.6 - 2.5	67/685 (9.8 %) CI 95 7.7 - 12.3	40/668 (6.0 %) CI 95 4.3 -8.1	115/686 (16.8 %) CI 95 14.0 -19.8
	Paper II	4/315 (1.3 %) CI 95 0.4 - 3.2	38/316 (12.0 %) CI 95 8.7 - 16.1	7/315 (1.6 %) CI 95 0.9 - 4.5	49/316 (15.5 %) CI 95 11.7 - 20.0
	Paper III	0/15 (0 %) CI 95 0 - 21.8	0/15 (0 %) CI 95 0 - 21.8	2/15 (13.3 %) CI 95 0.2 - 40.5	2/15 (13.3 %) CI 95 0.2 - 40.5
	Total	**13/1000 (1.3 %) CI 95 0.7 - 2.2**	**105/1016 (10.3 %) CI 95 8.5 - 12.4**	**49/998 (4.7 %) CI 95 3.7-6.4**	**166/1017 (16.3 %) CI 95 14.1 - 18.7**
field voles	Paper I	10/246 (4.1 %) CI 95 2.0-7.4	4/249 (1.6 %) CI 95 0.4 - 4.1	9/246 (3.7 %) CI 95 1.7 - 6.8	23/249 (9.2 %) CI 95 6.0 - 13.5
	Paper II	0/83 (0 %) CI 95 0 - 4.3	0/83 (0 %) CI 95 0 - 4.3	4/83 (4.8 %) CI 95 1.3 - 11.8	4/83 (4.8 %) CI 95 1.3 - 11.8
	Paper III	-	-	-	-
	Total	**10/329 (3.3 %) CI 95 1.5 - 5.5**	**4/332 (1.2 %) CI 95 0.3 - 3.1**	**13/329 (4.0 %) CI 95 2.1 - 6.7**	**27/332 (8.1 %) CI 95 5.4 - 11.6**
water voles	Paper I	2/29 (6.9 %) CI 95 0.9 - 22.1	1/30 (3.3 %) CI 95 0.08 - 17.2	0/30 (0 %) CI 95 0 - 11.6	3/30 (10 %) CI 95 2.1 - 26.5
	Paper II	0/1 (0 %)	0/1 (0 %)	0/1 (0 %)	0/1 (0 %)
	Paper III	-	-	-	-
	Total	**2/30 (6.7 %) CI 95 0.8 - 22.1**	**1/31 (3.2 %) CI 95 0.08 - 16.7**	**0/31 (0 %) CI 95 0 - 11.2**	**3/31 (9.7 %) CI 95 2.0 - 25.8**

ELISA = Enzyme Linked Immunosorbent Assay

Field voles and water voles were captured at a total of 42 trapping sites, but signs of infection could only be found at nine locations. At five of these nine sites presence of common voles could be confirmed, although the captured common vole individuals were TULV-negative at some locations. Only at four of 21 sites without detectable common vole presence, indications for TULV infection

(antibodies or viral RNA) could be confirmed in three field voles and two water voles. Because common vole populations can experience dramatic population crashes after high abundances, presence of this species beyond detection level at these sites cannot be excluded. Interestingly, for two of three seropositive field voles and both seropositive water voles from those four sites TULV-RNA amplification failed. The same RT-PCR method used for TULV-RNA amplification could have detected other vole-associated hantaviruses previously described in Germany such as PUUV and a strain of TATV (Traemmersee virus) (e.g., Reil et al., 2015; Binder et al., 2020b; Drewes et al., 2017; Jeske et al., 2019) but all sequences from the analyzed common voles, field voles and water voles could be identified as TULV.

In general, RNA detection was much more frequent in common voles than other species. Only 13 common voles (1.3 %) were seroreactive but RT-PCR-negative. The number of individuals with anti-TULV-IgG antibodies but no viral RNA was nearly 3 times higher for field voles and more than 5 times higher for water voles (Paper I, Table 1; Table 2), indicating virus clearance instead of establishment of persistence in non-reservoir voles.

4.2. Evolutionary host lineage and genetic structuring of TULV in Central Europe

Common voles survived the last glacial period in different refugia where populations were isolated from each other and re-colonized Europe proceeding from there, resulting in different evolutionary lineages of common voles in Europe: Western, Italian, Central and Eastern lineage (Heckel et al., 2005). Three of them could be found in the studied part of Europe. Common voles trapped in Luxembourg and France were belonging to the Western evolutionary lineage. Voles of the Central lineage represented the largest part of trapped animals in Germany, but in the eastern part (in the Federal State of Saxony) and as well as in Austria voles belonging to the Eastern lineage were trapped (Figure 2). Interestingly, in Saxony and the southern part of Brandenburg locations with Central and Eastern lineage were closely together, some of them separated by only a few kilometers (e.g., trapping sites 16 and 17, Figure 1, Paper I). At a single trapping site (Cunnersdorf in Saxony, number

27 on the map in Paper I (Figure I)) both lineages were sympatric. This surprisingly sharp geographic separation of evolutionary lineages may be caused by social structures in common vole communities that may lead to lower reproductive success of immigrants which could stabilize the genetic variance within a population and may prevent homogenization of allele frequencies between populations (Schweizer et al., 2007). Furthermore, females might prefer males of a certain lineage in hybrid zones (Beysard et al., 2014 and 2015).

TULV infection could be confirmed for voles of all three evolutionary lineages within the studied area. The detected partial S segment sequences were clearly distinguishable on small geographic scale (Paper I, Figure 2A and 2B). TULV clade I (Central North (CEN.N)) consists of two parapatric sister clades: Ia was identified in rodents from central and eastern Germany and Ib from northern, central, and western Germany. Clade II (Eastern North (EST.N)) can be found in eastern Germany and clustered with a strain isolated in Lodz, Poland. Clade III (Central South (CEN.S)) includes sequences from southern Germany, Luxembourg and the Alsace region in France as well as already published sequences from water voles from Switzerland. TULV sequences from trapping site number 27 in eastern Germany near the Czech border clustered with sequences from Austria and the TULV prototype strain Moravia from the Czech Republic in main cluster IV (Eastern South (EST.S)) (Paper I, Figure 4). At locations where RNA could be detected from more than one vole species, sequences from common voles and field voles or common voles and water voles clustered together, according to their location rather than showing differences according to different host animals.

In the eastern part of Germany, locations with different TULV clades (Ia and II) existed less than 10 km apart (Paper I, Figure 4, sites 16 and 17). This is in accordance to previously published findings of (genetic) isolation of common vole populations even at small geographic scale (Heckel et al., 2005; Schweizer et al, 2007, Beysard et al., 2015). Sequence identity was highest between the subclades Ia and Ib as well as Ia/Ib and II and lowest between Ia/Ib and III/IV with an average 8.9% sequence divergence on nucleocapsid protein level and 20.3% divergence in the analyzed part of the coding sequence of the S segment (Paper I, Tables 3A and 3B). Intra-cluster variability ranged from 5.8 –

11.9 % divergence in the partial S segment and 0.2 -0.5 % on nucleocapsid protein level. A similar range has been described for TULV nucleotide and amino acid sequences before. Especially when comparing sequences from different European countries differences of 7.7 – 19.6 % on S segment level and 0.3 – 6 % divergence of nucleocapsid protein level have been described (Scharninghausen et al., 2002; Sibold et al., 1999), but also within the same sampled rodent population TULV sequences may vary up to 7 % on nucleotide sequence level (Sibold et al., 1995). Further investigation of partial S, M and L segment sequences of all 4 monitoring sites in Germany showed that TULV sequences of all three segments from Gotha and Jeeser clustered in the CEN.N clade. Partial S, M and L segment sequences of the monitoring site Weissach clustered in the CEN.S clade. While the S and L segment sequences derived from the monitoring site Billerbeck clustered within CEN.S similar to Weissach, the M segment sequences clustered together with sequences from Jeeser and Gotha within CEN.N (Paper II, Figure S2 and Table 5). This may reflect different selective pressures based on the function of both glycoproteins as key components for viral entry compared to the other segments or might mark a reassortment in the wild. In general, reassortments are not rare events for hantaviruses. For PUUV in wild living bank voles, reassortment frequencies up to 32 % have been described (Razzauti et al., 2009). *In vitro*, reassortments with exchange of M segments but not S and L segments have been observed for PUUV and PHV as well as DOBV strains from striped field mice and yellow-necked field mice (Handke et al., 2010; Kirsanovs et al., 2010). For SEOV, PUUV and SNV intra-species exchanges of M segment could be observed in corresponding wild living host populations (Liu et al., 2012; Razzauti et al., 2009; Henderson et al., 1995).

Different TULV strains are able to infect several evolutionary lineages of the rodent host. TULV strains of EST.N clade was able to infect voles of the Central (C) as well as Eastern (E) lineage at trapping sites 16 (C), 21 (C), 30 (E) and 32 (E). At trapping site number 27 common voles of both Eastern and Central lineage were infected with TULV EST.S strains (Paper I, Figure 4). Further studies focusing on examining the association of TULV strains with specific evolutionary lineages of common voles came to more differentiated conclusions. When sampling different transects along the hybrid zone of Eastern and Central lineage along the Bavarian/Czech border, the transition between

different TULV clades was a remarkable eight times narrower than the hybrid zone between the host lineages (Saxenhofer et al., 2019). Host genetic factors might be responsible for that: More than 30 single nucleotide polymorphisms (SNPs) associated with immune system functions as well as membrane transport differed between Central and Eastern lineage, favoring the transmission of specifically adapted virus variants (Saxenhofer et al., 2022).

4.3. Common vole population dynamics and TULV prevalence

A total of 1487 common voles were trapped in spring, summer and autumn on grassland habitats in Germany at 4 locations: From 2010-2012 in Billerbeck and Weissach and from 2010-2013 in Jeeser and Gotha (Paper II, Figure 1). At each site one live trapping plot was paired with one snap trapping plot in three replicates. At every location, except Billerbeck, common vole presence could be detected every single year, although not continuously in every season.

The common vole abundances varied strongly between trapping locations, years as well as between seasons (Paper II, Figure 2). Estimated common vole abundance per location varied from 0 to 46 animals per 100 trap nights. Highest average abundance was 20 individuals per 100 trap nights, observed in Weissach in summer 2011 (Paper II, Figure 2). Populations usually peaked at the end of the reproductive period (autumn), although there were some exceptions. Summer peaks were seen in Weissach from 2010-2012, in Jeeser in 2010 and in Gotha in 2012 (Paper II, Figure 2).

Common vole abundance in the current season was negatively associated with TULV seroprevalence (Paper II, Table 2 and Figure 3a). This can be explained with the diluting effect of young, uninfected animals joining the population and has been described before for PUUV in bank vole populations (Adler et al., 2008; Kallio et al., 2010). While PUUV-infected females transfer protective maternal antibodies to their offspring (Kallio et al., 2010), which can be reflected in an increasing seroprevalence during the breeding season, TULV prevalence was so low at most sites (Paper II, Table 1) that a visible effect on seroprevalence was not to be expected. Thus, the growth of population

size during breeding period did not result in a direct increase in detection of TULV-reactive antibodies.

It was reported before that high prevalence of TULV seems to correlate with high abundances of common voles (Deter et al., 2008). In our study, vole abundance in the previous season was positively correlated with TULV prevalence in a delayed density-dependent manner (Paper II, Table 2 and Figure 3b) that has been previously described in longitudinal studies of SNV in deer mice (*Peromyscus* maniculatus) and PUUV in bank voles (Madhav et al., 2007; Mills et al., 1999; Olsson et al., 2010). Higher population densities result in increased contact probabilities between animals while high stress levels might impair the immune system making animals more susceptible during encounters (Navarro-Castilla et al., 2014, Adler et al., 2008).

While female common voles could be more frequently trapped, males were more frequently tested positive in ELISA and/or RT-PCR, but statistical significance of the sex-biased difference in infection varied. While in Paper I no statistical significance could be found ($\chi^2 = 2.319$, p-value=0.128), males in the monitoring study (Paper II) were statistically significantly more often tested positive for TULV-specific antibodies ($\chi^2 = 4.73$, $p = 0.03$). Literature is equally inconsistent. Several longitudinal studies report male-biased hantavirus infections in rodent reservoirs for SNV and PUUV (e.g., Schönrich et al., 2008; Douglass et al., 2001), possibly due to higher mobility of adult males, more contacts and higher aggression and subsequent transmission via biting (e.g., Escutenaire et al., 2002; Kozakiewicz et al., 2007; Easterbrook et al., 2007 and 2008). SEOV-infected male Norway rats have been found to have more viral RNA in spleen tissue (Klein et al., 2001) and were more likely to shed virus in saliva or through multiple routes such as saliva, urine and feces (Klein et al., 2001) while SEOV infection simultaneously increased their aggressive behaviour (Klein et al., 2004) which might explain a sex-biased difference in infection rates. In contrast, other studies found females to be more susceptible to PUUV infection (Kallio et al., 2006a) or no difference at all for PUUV and TULV (Escutenaire et al., 2000b; Schmidt-Chanasit et al., 2010). Deter et al., 2008 reported a male-biased PUUV infection for bank voles but no sex-related difference for TULV infection in common voles. Thus, the influence of

sex-specific differences on hantavirus prevalence is not yet conclusively clarified and needs further research. It is possible that underlying confounding factors leading to those differing conclusions are not yet identified.

Several studies suggested that rodent associated hantaviruses such as PUUV and SNV need a certain abundance threshold to be maintained (Tersago et al., 2008, Escutenaire et al., 2000; Luis et al., 2015). Populations with such extreme abundance changes as common voles may not be able to sustain pathogen infections for long because after population collapse pathogens cannot spread effectively due to the lack of susceptible hosts. While we found some gaps in TULV detection at all trapping sites at times of low abundance (Table 3; Paper II, Table 1), the virus always reappeared, either re-introduced by immigration or just below detection level at the previous trapping. Immigration into other populations is a common behaviour for male as well as female common voles (Schweizer et al., 2007) and this species was shown to easily repopulate territories within 3 months when the previous population has been nearly wiped out (Hein and Jacob, 2003).

The common vole is a short-lived species. While a maximum lifespan of nearly three years has been reported for healthy common voles in captivity (Devevey and Christe, 2009), life expectancy is markedly reduced in the wild to a mean of 4.5 months (Jacob and Brown, 2000). We also observed a high turnover in common vole populations, most marked individuals could not be recaptured 3 months later: only 3.3 % were caught in the following season in Jeeser, 4.5 % in Gotha, 2.5 % in Weissach and none in Billerbeck. Five individuals from Gotha could be captured 6 months after they were initially marked (0.9 %) as well as one female in Jeeser (0.4 %). Two females from Gotha even survived 9 months (0.4 %). Recaptures were more likely when high numbers of individuals had been captured in the previous season (Table 3).

The number of recaptures seems low compared to other vole species. In Belgium, recapture rates of 8.4 - 45.5 % between seasons have been reported for bank voles (Tersago et al., 2011b), in France and Poland almost all bank voles were recaptured a second or third time within two to six months

during the breeding season, indicating they might survive longer than the average common vole (Augot et al., 2008, Boratynski et al., 2009). Avoidance of traps due to previous handling seems unlikely as explanation for the observed low numbers of common vole recaptures since almost half (44.1 %) of all marked individuals could be trapped more than once during the three consecutive days each plot was sampled.

Instead, other factors are likely to contribute such as agricultural land use (Jacob, 2003; Bonnet et al., 2013), weather (Imholt et al., 2011; Esther et al., 2014) or predation by several different species (Ryszkowski et al., 1973; Paz et al., 2013). The observed peaks in summer instead of autumn at some locations might also be influenced by some of these factors. For example, mowing for hay production might affect common vole populations either directly by killing individuals or indirectly by damaging burrow structures and reducing the protective vegetation cover.

Table 3 Recaptures of individuals marked in the previous season(s)

	Number of re-captured marked/total number of trapped common voles			
	Jeeser	Gotha	Billerbeck	Weissach
Spring 2010	0/18	0/25	0/1	no captures
Summer 2010	1/93	1/67	0/3	0/76
Autumn 2010	7/60	3/112	0/18	0/17
Spring 2011	0/2	1/27	0/1	no captures
Summer 2011	no captures	0/38	no captures	0/138
Autumn 2011	0/26	4/106	0/8	8/76
Spring 2012	1/7	6/22	0/1	no captures
Summer 2012	0/8	3/72	no captures	0/15
Autumn 2012	0/31	6/62	no trapping	0/3
Spring 2013	no captures	no captures	no trapping	no trapping
Summer 2013	0/2	no captures	no trapping	no trapping
Autumn 2013	0/23	0/4	no trapping	no trapping
total	**9/270**	**24/535**	**0/32**	**8/325**

No captures = trapping was conducted, but common voles were not captured; no trapping = no trapping conducted

Twenty-six recaptured common voles could be sampled twice, one animal even three times. More than two thirds (69 %) did not change their serological status. Blood samples from 7.7 % were less reactive in ELISA than in the previous season- changing from equivocal to negative or from positive to

equivocal. All these animals were adults at the time of their first capture, so loss of maternal antibodies is not a likely explanation. Seroreversion from positive to negative ELISA results could not be observed. Another 7.7 % reacted slightly more strongly (equivocal result instead of negative) at the second analysis three months later. For 14.3 % of common voles seroconversion was shown, all of them seroconverted within three months. Compared to other studies on Cricetidae-associated hantaviruses the observed rate of seroconversion was low. Imitating the natural infection route with PUUV via intranasal inoculation, 50 % of bank voles seroconverted in a laboratory setting (Hardestam et al., 2008) within 42 days. Studies on SNV even found 33 % - 85 % seroconversion of wild living cricetid hosts within three to six months (Abbott et al., 1999; Suzán et al., 2009).

The reported maximum TULV RNA prevalence here was 37.5 %. PUUV, another European hantavirus carried by voles, may reach up to 80-100 % prevalence, especially in older, overwintered animals (Vaheri et al., 2021; Weber de Melo et al., 2015). Infected host animals shed as early as 5 days post infection and presumably their whole life in varying amounts (Vaheri et al., 2013b). Shedding for up to 15 months has been observed for PUUV infected bank voles (Bernshtein et al., 1999). Shorter lived animals might shed less virus between infection and death and thus, the observed low life expectancy might contribute to the low over all prevalence of TULV in common vole populations.

4.4. Multiple pathogens and coinfections in common voles and other rodent species

4.4.1. Cocirculation of different pathogens

In a pilot study in Austria common voles and other rodents were collected and investigated not only for TULV and other hantaviruses, but also for CPXV, LCMV, bacterial zoonotic pathogens such as *Leptospira* spp., *Borrelia afzelii*, *Coxiella burnetii*, *Rickettsia* spp., and *Bartonella* spp. as well as endoparasites (*Toxoplasma gondii*). Of particular interest were coinfections and their incidence in common voles compared to other species.

Trapping efforts at five rural sites in the municipality Laa an der Thaya and two rural sites in the municipality Altenburg in northern Austria resulted in a total of 110 small mammals trapped belonging to 4 different species: Common voles (n = 15), yellow-necked field mice (n =29), wood

mice (n = 26) and bank voles (n = 40). In total, 43.6 % (n = 48) of all 110 analyzed rodents were positive for at least one pathogen in either serology and/or molecular detection methods (Table 4). There was no trapping site without pathogen detection in small mammals (35-55.5 % positive animals per site).

The pathogen most frequently detected in common voles was *B. afzelii* (46.7 %), followed by *Rickettsia* spp. (33.3 %), *Bartonella* spp. (20 %), TULV (13.3 %) and *Leptospira* spp. (6.7 %) (Table 4). While neither LCMV, CPXV nor *C. burnetii* could be detected in common voles in this study, all three pathogens have been found in association with common voles before (e.g., Kallio-Kokko et al., 2006; Tagliapietra et al., 2009; Essbauer et al., 2009; Fischer et al., 2020; González-Barrio et al., 2021). The results of this pilot study show that common voles are particularly susceptible to a variety of zoonotic pathogens. In total, 66.7 % of common voles showed serological or molecular evidence of infection, followed by wood mice (57.7 %), bank voles (35 %) and yellow-necked field mice (34.5 %).

We could identify 4 different species of *Bartonella* spp.: *B. taylorii*, *B. birtlesii*, *B. grahamii and B. doshiae.* Common voles were found to be associated with one specific species (*B. taylorii*). This pathogen seems to be able to successfully infect a wide variety of hosts and was the pathogen most often detected with regard to all four analyzed rodent species. But while we detected co-circulation of different *Bartonella* species at the same trapping site we did not find any coinfection of host animals with different species. Animals can be re-infected by another species shortly after they cleared the previous *Bartonella* spp. infection, but coinfections seem to be uncommon (Birtles et al., 2001).

Both tick-borne pathogens *B. afzelii* and *Rickettsia* spp. are considered emerging threats in Europe (Vandekerckhove et al., 2021; Parola et al., 2013) and have been detected in ticks all over Austria with varying prevalences (25 – 33.9 % and 5.7 % - 50 %, respectively) (Dobler et al., 2008; Schött et al., 2017). Rodents in Austria are less well studied. With up to 100 human cases per 100,000 inhabitants per year (Rizzoli et al., 2011) and 11.6 hospitalized patients / 100,000 cases per year

(Stiasny et al., 2021), Lyme borreliosis is a major public health concern in Austria. To our knowledge this is the first detection of *B. afzelii* in Austrian rodents. Common voles might play an important role in the infection cycle of *Borrelia* spp. It could be shown that they efficiently transmit *B. burgdorferi* sensu lato to ticks feeding on them (Radzijevskaja et al., 2013) and that common voles were more often infested by immature *Borrelia*-positive castor bean tick (*Ixodes ricinus*) larvae compared to yellow-necked field mice and bank voles (Sinski et al., 2006).

Table 4: Detection of zoonotic pathogens in common voles and other species in the pilot study in Austria

Pathogen	Detection method	Common voles	Bank voles	Yellow-necked field mice	Wood mice
Viruses					
Orthohantaviruses	ELISA	**2/15**	0/40	**1/29**	0/26
	RT-PCR	**2/15**	-	0/29	0/26
Cowpox virus	IFA	0/15	**1/40**	0/29	0/26
	PCR	0/15	0/40	0/29	0/26
Lymphocytic choriomeningitis mammarenavirus	IFA	0/15	0/40	0/29	**1/26**
	RT-PCR	0/15	0/40	0/29	0/26
Bacteria					
Leptospira spp.	PCR	**1/15**	**3/40**	**2/29**	**2/26**
Borrelia afzelii	PCR	**7/15**	**4/40**	**2/29**	**1/26**
Coxiella burnetii	PCR	0/15	0/40	0/29	0/26
Rickettsia spp.	IFA	**3/15**	**4/40**	**2/29**	**2/26**
	PCR	**2/15**	**1/40**	0/29	0/26
Bartonella spp.	PCR	**3/15**	**3/40**	**4/29**	**11/26**
Endoparasite					
Toxoplasma gondii	PCR	0/15	0/40	0/29	0/26
Total individuals tested positiv		**10/15**	**14/40**	**10/29**	**15/26**

IFA = Immunofluorescence assay

Human risk for infection with spotted fever *Rickettsia* in Austria needs further evaluation but a seroprevalence of 7.7 % in blood donors from Tyrol indicates this disease might be underreported (Sonnleitner et al., 2013). While no further data is available concerning *Rickettsia* spp. in voles in Austria, common voles have been described as reservoir for different *Rickettsia* species in Germany with varying prevalence of 0 – 5.7 % (Fischer et al., 2018a).

With more than one third of common voles infected with either pathogen, they might be an important amplification host and surveillance measurements should be considered since they are known to undergo cyclic population density changes with extremely high abundances (Klemola et al., 2000; Bryja et al., 2005). Higher abundances mean more opportunities for questing ticks to encounter hosts and therefore higher transmission odds between vertebrate amplification host and arthropod vector (Tkadlec et al., 2019).

Leptospira spp. prevelance of 6.7 % in common voles seems surprisingly low but is in accordance with the reported prevalence of 6.4 % in European hare (*Lepus europaeus*) also caught in northeastern Austria (Winkelmayer et al., 2005). *Leptospira* spp. prevalence seems highly variable according to sampled location. A study in Spain for example reported a similarly low prevalence of 7.9 % in common voles (Jeske et al., 2021b), prevalence in Lithuanian common voles varied between 0 – 13.3 % (Jeske et al., 2022), while studies from Germany report *Leptospira* spp. detection in up 30 % of analyzed common voles (Fischer et al., 2018b). Prevalence may also depend on season. Jeske et al., 2021(a) reported an increase in *Leprospira* prevalence in common voles from spring (5.1 %) to autumn (40.6 %) in Central Germany. Even at the presented low prevalence the threat to human health should not be underestimated. Outbreaks of leptospirosis are known to be associated with heavy rainfall (Radl et al., 2011; Desai et al., 2009) and flooding events - weather conditions that may become more common as climate changes (Madsen et al., 2014).

4.4.2. Coinfections

Again, common voles were the species most susceptible: 66.7 % of common voles infected with one pathogen were coinfected by at least one other, compared to 6.9 % of yellow-necked field mice and 2.5 % of bank voles. No coinfection could be detected in wood mice. Common voles were the only species where simultaneous infections with three pathogens were detected. Usually, mammals with short life expectancy such as common voles invest less in immunological functions, especially the

adaptive immune response, which could make them more susceptible to a wide variety of pathogens and multiple infections they could pass on to humans (Vaumourin et al., 2015).

Pathogen-host interaction is thought to be fine-tuned to optimize fitness for the pathogen without killing the host they depend on (Alizon and van Baalen et al., 2008). This involves modulation of the host's immune system but also behavioural changes such as increased/decreased mobility, altered social preferences to enhance contacts to other susceptible animals or loss of fear of natural predators to ensure uptake by their final host to complete their reproductive cycle (Hafer et al., 2016). This balance is disturbed when two or more pathogens are competing for the same resources (the host), trying to optimize their surroundings according to their own needs. Coinfection may even select for pathogen variants with higher virulence compared to specimens that do not have to compete (Alizon and van Baalen et al., 2008).

Both TULV infected common voles were coinfected with either *B. afzelli* or *B. afzelii* and *B. taylorii*. All *Bartonella* spp.-positive common voles were also coinfected. For *B. afzelii*- and *Rickettsia* spp.-positive common voles single infections could be detected. Interestingly, common voles were either positive for *Rickettsia* spp. DNA or *Rickettsia* spp.-specific antibodies but not both. The majority of common voles showing evidence of past or present infection with *B. afzelii*- and *Rickettsia* spp. was infected with at least one additional pathogen (71 % and 60 %, respectively) (Paper III, Table 1 and Table 4). No coinfection could be detected for the *Leptospira* spp. positive common vole individual.

Unfortunately, literature concerning coinfection of the zoonotic pathogens studied here is scarce, but coinfections of hantaviruses (DOBV and TULV) and *Leptospira* spp. are regularly described when both are analyzed in captured rodents (Tadin et al., 2012 and 2016, Jeske et al., 2021a) as well as coinfections of hantaviruses and *Bartonella* spp. (Tadin et al., 2016). The prevalence of coinfection of common voles with TULV and pathogenic *Leptospira* species seems to be dependent on several individual as well as population level factors (e.g., age, vole population density, prevalence of TULV in vole population, prevalence of *Leptospira* spp. in vole population). Coinfections were especially

common at locations were *Leptospira* spp. prevalence exceeded 35 % which might explain the absence of coinfections in the pilot study in Austria. Similar to TULV, the common vole abundance in the previous season influences the prevalence of these coinfections but not in a linear but exponential manner (Jeske et al., 2021a).

Coinfection of several *Bartonella* species and *Borrelia* spp. has also been described before in bank voles with no preference for a specific coinfecting *Bartonella* species (Buffett et al., 2012). Interestingly, other pathogen prevalences also change in fluctuating common vole populations. *Bartonella rochalimae* prevalence for example varied similar to TULV with current and previous vole density (delayed-density dependence) (Rodriguez-Pastor et al., 2019). This is of special interest since *Bartonella* spp. has been shown to interact synergistically with orthohantaviruses (SNV). Co-infected animals had an altered innate immune response and lower antibody titers compared to animals infected with either pathogen alone (Lehmer et al., 2018).

While the observed 8.2 % of animals tested positive for more than one pathogen in this pilot study seems to be low, it should be considered that this study as well as most other studies was looking for a very limited number of pathogens. Important groups like helminths that may have no direct importance for human public health but may influence the host's susceptibility to and carrier ability of pathogens (Kamiya et al., 2018; Behnke 2009) are usually neglected. This could explain why some studies reported a decreased survival of hantavirus infected rodent hosts (e.g., Kallio et al., 2007; Tersago et al., 2011a; Douglass et al., 2001; Luis et al., 2012) while others did not (e.g., Meyer and Schmaljohn, 2000; Bernstein et al. 1999).

Coinfection may lead to higher shedding and consecutive spillover of putative zoonotic agents to humans. For example, bat paramyxoviruses and *Hendra henipavirus* spillover from bats to humans (Peel et al., 2019) or greater bacterial load and slower decline of *Bartonella* spp. load in hosts that are co-infected with *Mycobacterium tuberculosis* (Eidelmann et al., 2019).

Since coinfections may change the clinical picture of diseases in humans and may lead to more severe disease outcomes and/or misdiagnosis (Vamourin et al., 2015) they are an important factor for human health, considering than an estimated 30 % or more of human infections are coinfections (Vamourin et al., 2015; Alizon et al., 2013). When analyzing samples from patients hospitalized with severe leptospirosis in Sri Lanka, coinfection of *Leptospira* spp. and hantaviruses could be confirmed for 22.5 % of current patients and 26 % of patients in retrospective analysis (Sunil-Chandra et al., 2015). Future studies should focus more on evaluating the prevalence and impact of multiple infections in both animal hosts as well as humans.

5. OUTLOOK

It could be shown that TULV is a very common and wide-spread infection in European common voles of different evolutionary lineages. Average TULV prevalence is low compared to other orthohantaviruses and varied with trapping location and season. While the capture-mark-recapture study did not detect TULV-infected common voles continuously, the virus always re-appeared in following trapping season. Therefore, future studies should repeat trapping efforts at sites where no TULV could be detected in summer or autumn when higher population densities are to be expected to confirm this result. Live trapping instead of snap trapping should be considered as standard method since this method has no influence on the local population because animals can be released after sampling. Should it be necessary to remove animals to obtain specific samples (e.g., lung tissue for hantavirus RT-PCR diagnostic), the target species can be killed selectively, all other species can be released. Furthermore, the trapping success is higher, increasing the chances of detecting infections of low prevalence. RT-PCR was the most reliable detection method and should be used for monitoring studies rather than serological methods whenever possible.

Although common voles seem to be the best suited reservoir host for TULV, spillover events to other vole species were not rare. The higher mutation frequency of RNA viruses compared to DNA viruses may facilitate adaption to a new host. Therefore, further research should evaluate the host association of TULV to reveal possible adaption processes and host factors involved in species barriers. *In vitro* studies in different cell lines of different vole species could help to pinpoint the requirements for susceptibility of (spillover) infection as well as adaption processes of the virus after initial entry. Several vole cell lines are already existing (e.g., from different common vole, bank vole, and field vole tissues), ready to be used in future research. More cell lines of other species (e.g., for investigation of water voles) or cell lines of further tissues are needed, but the workflow for their generation is well established. Furthermore, animal infection experiments will help to find out consequences of TULV infection, and coinfections with other pathogens to the common vole reservoir.

TULV sequences as well as the evolutionary lineages of the rodent host exhibit a distinct genetic structuring throughout the studied area although hybrid zones where different lineages meet have been described before and could be confirmed at one trapping site in Saxony. Those hybrid zones might be an interesting opportunity to study host-pathogen interaction and evolving genetic barriers of parasite transmission. Already performed transect studies in those hybrid zones should be repeated regularly to detect possible changes and adaptation processes and prove the potential adaption processes in other common vole-specific pathogens, e.g., common vole hepevirus or polyomavirus.

Rodents are now the dominant mammal clade in most environments, making their carried zoonotic agents of greater importance to human health than ever. Of all tested species, common voles were the most susceptible to infection with the tested emerging zoonotic pathogens and they had the greatest capability to host several pathogens at once. This is worrisome, because they can reach extremely high population densities and are superior to other species in surviving in extensively used agricultural landscapes. Therefore, future investigations should focus not only on TULV, but also other common vole-associated zoonotic and currently non-zoonotic pathogens.

Not only TULV but also several other pathogens such as *Bartonella* spp. and *Leptospira* spp. have been shown to fluctuate with common vole population density, emphasizing the importance of this species as reservoir for emerging zoonotic pathogens. Adding the fact that the effects of coinfections in wild living rodent populations (e.g., on shedding) are not well studied at the moment, the risk they pose cannot be underestimated. Especially with regard to climate change which will re-form ecosystems world-wide in the next years and centuries with habitat changes, restructuring of animal communities and loss of species diversity, favoring generalist species such as common voles. They are versatile and can quickly populate open grassland habitats as pioneer species which means even efforts to fight the climate change such as renaturation of surface mines might open new opportunities for common voles and facilitate their dispersal to new areas. Future research projects assessing the risk for human health should therefore consider surveillance programs for wild

common vole populations and should include further evaluation of the effects of simultaneous infection of rodents with several pathogens on the host as well as the consequences for humans exposed to those reservoirs in a One Health framework.

6. SUMMARY

More than 1400 species pathogenic to humans are currently known, 61 % of them are zoonotic-transmitted from animals to humans. Hantaviruses are emerging zoonotic pathogens. Those viruses have a single stranded RNA genome and have been detected in several mammal species and recently in fishes and one reptile species. One of the hantaviruses that have been detected in Germany is the *Tula orthohantavirus* (TULV) that has also been described in several countries other in Europe and Asia. The first isolation was from common voles (*Microtus arvalis*) which are assumed to be the primary host, but since then TULV infections have been detected in several related rodent species. Descriptions of human infections with TULV are rare.

The aim of this work was to characterize the distribution of TULV in European common vole populations, to clarify the host association of TULV and to investigate correlations between host population dynamics and changes in TULV prevalence. Furthermore, the potential of common voles as reservoir for other rodent-borne pathogens was examined in comparison to other rodent species.

Molecular and serological analysis of rodents captured at 87 locations in Germany, France, Luxembourg, and Austria revealed TULV infections at 53.6 % of all trapping locations. The seroprevalence in common voles was low with a mean of 8.5 % (range: 0 – 19 %). TULV RNA was more often detected (mean: 15.3 %, range 0 - 37.5 %). Field voles (*Microtus agrestis*) and water voles (*Arvicola amphibius*) were less often tested positive for TULV: mean seroprevalence was 7 % for field voles and 6.7 % for water voles. RNA could be detected in 5.4 % of all tested field voles and 3.2 % of water voles and with exception of a single field vole only when TULV-RNA-positive common voles were trapped at the same location. Those results indicate that TULV infections of field and water voles are spillover infections from sympatric TULV-infected common voles. Phylogenetic analysis revealed distinct genetic differences between TULV sequences of regions of greater geographical distance which were associated with different evolutionary common vole lineages. Furthermore, we could detect genetic differences between TULV strains from trapping sites close to each other (ca. 10 km).

In a capture-mark-recapture study 1042 common voles captured in live traps in Germany were sampled as well as 225 captured in snap traps. When analyzing the seroprevalence of fluctuating common vole populations over several years and seasons we found a negative correlation between prevalence and population density in the current season but a delayed density-dependent positive correlation between the current population density and seroprevalence in the next season. However, this trend varied geographically between the four trapping locations. Usually, population density as well as seroprevalence peaked at the end of the reproductive period in autumn with the exception of Weissach (2010-2012), Jeeser (2010) and Gotha (2012) where population peaks in summer were observed.

In a pilot study in Austria common voles were captured as well as three other rodent species. They were investigated not only for presence of different viruses (TULV, *Dobrava- Belgrade orthohantavirus* (DOBV), *Puumala orthohantavirus* (PUUV), *Lymphocytic choriomeningitis mammarenavirus* (LCMV), Cow*pox virus* (CPXV)) but also pathogenic bacteria and endoparasites (*Leptospira* spp., *Toxoplasma gondii*, *Borrelia afzelii*, *Coxiella burnetii*, *Rickettsia* spp. und *Bartonella* spp.). Of all four captured species, common voles were most often infected with at least one pathogen (66.7 %), followed by wood mice (*Apodemus sylvaticus*) (57.7 %), bank voles (*Myodes glareolus*) (35 %) and yellow-necked field mice (*Apodemus flavicollis*) (34.5 %). Common voles were also exceptionally susceptible to multiple infections: 66.7 % of them were infected with two or three different pathogens, compared to 6.9 % of yellow-necked field mice and 2.5 % of bank voles. No multiple infections could be detected in wood mice.

The broad geographic distribution of TULV in its reservoir host is in contrast to the rare reports of human infection but might be explained with a low pathogenicity for humans or with the low prevalence in host populations. In addition, the rare detection of human TULV infections could be a result of the used diagnostic methods. Since the reservoir population is known for its dramatic changes in population density and recurring superabundances which facilitates frequent contact to humans, TULV should more often be considered as cause for human disease in future analysis. In

addition, several other zoonotic pathogens could be detected in common voles which could influence TULV infections in the reservoir host but also TULV transmission to humans and therefore deserve more attention in future research.

7. ZUSAMMENFASSUNG

Aktuell sind mehr als 1400 humanpathogene Krankheitserreger bekannt, von denen 61 % zoonotisch sind, also vom Tier auf den Menschen übertragen werden können. Zu den Zoonoseerregern, die eine zunehmende Gefahr für den Menschen darstellen, gehören die Hantaviren. Diese Viren besitzen ein einzelsträngiges, segmentiertes RNA-Genom und sind in einer Vielzahl von Säugetierwirten, kürzlich aber auch in Fischen und einem Reptil nachgewiesen worden. Eines der Hantaviren, die in Nagetieren in Deutschland nachgewiesen werden konnte, ist das *Tula orthohantavirus* (TULV), das außerdem bereits in vielen verschiedenen europäischen Ländern und Teilen Asiens beschrieben wurde. Die Erstisolation erfolgte aus der Feldmaus (*Microtus arvalis*), diese gilt als primäre Wirtsspezies. Jedoch konnten Infektionen inzwischen auch in anderen verwandten Nagetierarten nachgewiesen werden. Bisher wurden nur wenige humane TULV-Infektionen beschrieben.

Das Ziel dieser Arbeit bestand darin, die Verbreitung von TULV in europäischen Feldmauspopulationen zu charakterisieren, die Wirtsassoziation zu prüfen und mögliche Zusammenhänge zwischen Veränderungen in Wirtsspezies-Populationen und TULV-Prävalenz zu untersuchen, sowie das Auftreten von anderen Nagetier-übertragenen humanpathogenen Krankheitserregern in Feldmäusen und anderen Nagetierarten zu analysieren.

Molekulare und serologische Untersuchungen von Nagetierfängen an 87 Standorten in Deutschland, Frankreich, Luxemburg und Österreich zeigten für 53,6 % der Fangorte TULV-Infektionen. Die Seroprävalenz unter Feldmäusen war niedrig und lag im Durchschnitt bei 8,5 % (0 - 19 %). TULV-RNA konnte dagegen häufiger detektiert werden. Hier schwankte die Prävalenz zwischen 0 – 37,5 % (im Mittel: 15,3 %). Seltener wurde TULV in Erdmäusen (*Microtus agrestis*) und Schermäusen (*Arvicola amphibius*) nachgewiesen. Die durchschnittliche Seroprävalenz betrug bei Erdmäusen 7 % und bei Schermäusen 6,7 %. TULV-RNA konnte in 5,4 % der Erdmäuse und 3,2 % der Schermäuse nachgewiesen werden und mit Ausnahme einer einzelnen Erdmaus ausschließlich dann, wenn am

gleichen Fangort auch TULV-RNA-positive Feldmäuse gefangen wurden. Diese Untersuchungsergebnisse deuten bei den Erd- und Schermäusen auf Spillover-Infektionen hin, deren Ursprung sympatrisch vorkommende TULV-infizierte Feldmäuse darstellen. Die phylogenetische Analyse zeigte deutliche genetische Unterschiede zwischen TULV-Sequenzen von entfernten Regionen, die auf eine Assoziation mit unterschiedlichen evolutionären Linien der Feldmaus zurückzuführen sind. Darüber hinaus wurden auch genetische Unterschiede zwischen TULV-Stämmen von Fangorten mit geringen Entfernungen von ca. 10 km beobachtet.

Bei Rückfangstudie (Fang-Markierung-Wiederfang) an vier Standorten in Deutschland wurden von insgesamt 1042 in Lebendfallen gefangenen Feldmäusen und von weiteren 225 mit Schlagfallen gefangenen Feldmäusen Proben zur Analyse gewonnen. Verfolgt man die Entwicklung der Seroprävalenz in sich ändernden Feldmauspopulationen über mehrere Jahre und verschiedenen Zeitpunkten im Jahr, so waren Populationsdichte und Seroprävalenz innerhalb einer Jahreszeit negativ korreliert. In der nachfolgenden Fangsaison gab es jedoch einen positiven, verzögert dichteabhängigen Zusammenhang zwischen der aktuellen Seroprävalenz und der Populationsdichte der Vorsaison. Dieser Trend zeigte jedoch räumliche Variabilität, da sich die vier Standorte in ihrer Ausprägung unterschieden. Sowohl Populationsdichte als auch Seroprävalenz erreichten ihren Höhepunkt zumeist zum Ende der Reproduktionsperiode im Herbst. Ausnahmen bildeten der Fangort Weissach (2010-2012) sowie die Fangorte Jeeser (2010) und Gotha (2012). Hier wurde die maximale Populationsdichte bereits im Sommer erreicht.

In einer Pilotstudie wurden Feldmäuse und drei weitere Nagetierarten gefangen und auf verschiedene Viren (TULV, *Dobrava-Belgrad Orthohantavirus* (DOBV), *Puumala Orthohantavirus* (PUUV), *Cowpoxvirus* (CPXV) und *Lymphozytäres Choriomeningitis-Mammarenavirus* (LCMV)), humanpathogene Bakterien und Endoparasiten (*Leptospira* spp., *Toxoplasma gondii*, *Borrelia afzelii*, *Coxiella burnetii*, *Rickettsia* spp. und *Bartonella* spp.) untersucht. Im Vergleich aller vier gefangenen Spezies waren vor allem Feldmäuse mit mindestens einem Erreger infiziert (66,7 %), gefolgt von 57,7 % der Waldmäuse (*Apodemus sylvaticus*), 35 % der Rötelmäuse (*Myodes glareolus*) und 34,5 % der

Gelbhalsmäuse (*Apodemus flavicollis*). Auch was Infektionen mit mehr als einem Erreger betraf, waren Feldmäuse besonders betroffen: 66,7 % von ihnen waren mit zwei oder drei verschiedenen Krankheitserregern infiziert, im Vergleich dazu traf das nur bei 6,9 % der Gelbhalsmäuse und 2,5 % der Rötelmäuse zu. In Waldmäusen konnten keine Mehrfachinfektionen nachgewiesen werden.

Die große geografische Verbreitung von TULV im Reservoir steht im Gegensatz zu den bisher nur sehr selten beschriebenen humanen Infektionen, kann aber möglicherweise mit einer geringen Pathogenität des Virus oder mit der relativ geringen Prävalenz in den Reservoirpopulationen erklärt werden. Der seltene Nachweis von humanen TULV-Infektionen könnte andererseits aber auch auf die bei Patienten üblicherweise verwendeten Diagnostikverfahren zurückgeführt werden. Zukünftige Untersuchungen sollten deshalb verstärkt auch TULV als mögliche Ursache humaner Infektionen in Betracht ziehen, zumal der Wirt, die Feldmaus, massiven Veränderungen der Populationsdichte unterliegt und bei Massenvermehrungen häufiger Kontakt zum Menschen möglich ist. Darüber hinaus wurden in der Feldmaus weitere Zoonoseerreger gefunden, die die TULV-Infektion im Reservoir und möglicher Weise die Übertragung auf den Menschen beeinflussen könnten, und deshalb zukünftig verstärkt in die Untersuchungen einbezogen werden sollten.

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9. APPENDIX

9.1. List of publications

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Schmidt S, Essbauer SS, Mayer-Scholl A, Poppert S, Schmidt-Chanasit J, Klempa B, Henning K, Schares G, Groschup MH, Spitzenberger F, Heckel G, Richter D, Ulrich RG. Multiple infections of rodents with zoonotic pathogen in Austria. Zoonotic and Vector Borne Diseases 2014; 14:467-75

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