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Hantavirus Infections in Host Populations of Yellow-Necked and Field Mice (Rodentia: Muridae) in South Bulgaria

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Abstract: The wood and field mice of the genus *Apodemus* are natural reservoirs of hantaviruses: *Dobrava*, *Puumala* and *Saarema* virus types. In the last few years, cases of hemorrhagic fever with renal syndrome were reported from different regions in Bulgaria, but the factors, influencing hantavirus transmission within host populations have not been well investigated. Sensitive molecular genetic methods (real time PCR) for detection of hantaviruses in wood and field mice, captured in endemic for the virus region in Southern Bulgaria, were used. *Dobrava* virus type (DOBV) was genetically determined in two *Apodemus* species: *Apodemus flavicollis* (Melchior, 1834) and *Apodemus agrarius* (Pallas, 1771). The overall DOBV prevalence was 7.7% for *A. flavicollis* and 1.43% for *A. agrarius*. A number of factors, influencing the association of DOBV infections in yellow-necked wood and field mice, were evaluated. The logistic regression model showed four factors in the model outcome: species, age, gender and reproductive status. This analysis highlights the importance of population demography in the successful circulation of hantaviruses. The overwintered reproductively active males of yellow-necked mice appear critical to the success of DOBV circulation and persistence within host populations in South Bulgaria.

Key words: Dovrava virus, *Apodemus flavicollis, Apodemus agrarius*

Introduction

Dobrava virus type (DOBV) is European hantavirus that causes hemorrhagic fever with renal syndrome (HFRS) and seems to be the most life-threatening hantavirus in Europe. Case-fatality rates in Balkan countries are as high as 12% (Gligich *et al.* 1992; Avsic-Zupanc *et al.* 1999; Papa, Antoniadis 2001). DOBV is hosted by two different rodent species, *Apodemus flavicollis* (yellow-necked wood mouse) and *A. agrarius* (striped field mouse) (Kamarinchev 1996; Sibold *et al.* 2001; Klempa *et al.* 2003). In the last few years, cases of HFRS were reported from different regions in Bulgaria (Kamarinchev 1996; Papa, Christova 2011), but the factors, influencing hantavirus transmission within host populations have not been well investigated. The prevention of the disease agent requires a detailed knowledge of the ecology and epizootiology of the hosts of zoonotic viruses.

The hantavirus transmission appears to be a function of many factors, including geographic area, climate change, types of biotope alterations and the Hantavirus species (Dearing, Dizney 2010). The demographic factors and the ecology of the hosts play a key role in hantavirus prevalence. A relation between the male gender and hantavirus prevalence was reported for numerous hantaviruses (Glass *et al*. 1988; Cantoni *et al*. 2001). The role of individual body mass and antibody prevalence has been demonstrated for several hantaviruses (Glass *et al*. 1988, Mills *et al*. 1999, Escutenaire *et al*. 2000), implying that these viruses are transmitted horizontally among hosts. Varying seasonal patterns in the prevalence of infection with hantaviruses in their host populations have also been reported (Niklasson *et al*. 1995, Escutenaire *et al*. 2000, Tersago *et al.* 2011). A positive relation between hantavirus prevalence and population density in deer mouse (*Peromyscus maniculatus*) and brush mouse (*Peromyscus boylii*) was reported by Begon *et al*. (2002) and Mills *et al*. (1999).

Knowing the population demographic factors associated with DOBV infection in their rodent hosts in Bulgaria is important because it leads to an understanding of the mechanisms of DOBV transmission and this knowledge, in turn, may lead to the improved prediction of the relative risk of HFRS for humans.

The focus of the present study was to relate and rank individual and population factors, influencing the prevalence of DOBV and determine which of these factors are essential for the spread of Hantavirus within a host population species.

Material and Methods

Rodent sampling and species abundance

Small-mammal trapping was done in the vicinity of Peshtera, an endemic for the HFRS region in Southern Bulgaria (42°033'- 42° 051'N, 24°310'- 24°550'E). The collection of animals took place for three nights each month, beginning in the spring of 2011 until the spring of 2012. Each month, ten 100 m-lines of 20 Sherman live-traps per 3 nights were set in the moist grassy, wooded and shrubby habitats. The species from genus *Apodemus* were monitored.

As a measure of relative abundance, trap success was calculated as the number of individuals captured per 100 trap nights.

Species identification

The morphological diagnostics of the species *Apodemus sylvaticus,* (Linnaeus, 1758) and *Apodemus flavicollis* (Melchior, 1834) in the south part of their genus range in Europe is heavily complicated by the morphological parallelism. The simple diagnostics of the species is hampered by the strong opposite clinal variability of the external size characteristics. The wood mice individuals were identified to the species *A. flavicollis* or *A. sylvaticus* on the basis of analysis of cytochrome oxidase c subunit I (COI) and cytochrome b (cytb) gene fragments. Tissues were submitted to the Canadian Centre for DNA Barcoding, Biodiversity Institute of Ontario, University of Guelph (Guelph, Canada) and standard barcoding protocols for PCR amplification and sequencing were employed, following PCR and sequencing with regular and M13-tailed universal vertebrate primer cocktails (Clare *et al*. 2007; Ivanova *et al*. 2006).

Hantavirus detection

TaqMan RT-PCR for the detection of hantaviruses in host species was used. Primers and probes described by Kramski *et al.* (2007) were applied. Reactions were conducted in 25 μl final volume and consisted of 12.5 μl QuantiTect Master Mix, 4.5 μl ddH_2O, 1 μl of each primer pair (Forward-Reverse), 0.25 μl probe, ROX Dye 0.5 μl, QuantiTect RT Mix 0.25 μl and 5 μl of extracted viral RNA.

Investigated individual and population factors associated with hantavirus transmission

The following data were recorded for each animal: species, age, gender and reproductive status. The age determination was done according to criteria of the degree of teeth wearing out (Adamczewska-Andzeewska 1973) and each animal was subsequently assigned to one of four age classes: juvenile (< 3 months of age), sub adult (between 3 and 5 months of age), adult (between 6 and 9 months of age) or senile (overwintered, > of 10 months of age). Males with scrotal testes and females with a perforate vaginal orifice, lactating, or with enlarged mammary glands were defined as reproductively active individuals. The phase of the population density was designated as "increase," "peak" or "decline", depending on the relative amplitude of trap success.

Multivariable logistic regression models in STATA were used to identify statistically significant characteristics. The risk factors of infection were studied at the individual and at the population level as well. The species, age, gender and reproductive status were investigated as explanatory factors. The DOBV status (PCR positive or PCR negative) was used as a dichotomous dependent variable. Rodent ID number was included as a random effect. The critical probability was $p<0.05$ throughout. The odds ratios (OR) were presented in favor of infection in relation to reference levels for nominal variables.

Results

Molecular-genetic species identification of the wood mice (subgenus Apodemus)

Based on the provided analysis of cytochrome oxidase c subunit I (COI) and cytochrome b (cytb) gene fragments, all captured individuals from the subgenus *Apodemus* were identified as yellow-necked mice (*A. flavicollis)*.

Trapping summary

Overall 7240 trap nights were accumulated. In all, 198 captures of *Apodemus* individuals were recorded from May 2011 to January 2012. Of these, 102 (51.5%) individuals were of the species *Apodemus agrarius,* and 92 (46.5%) were of the species *Apodemus flavicollis*. The striped field mouse (*A. agrarius*) prevailed in the moist grassy habitats, whereas the yellow-necked mouse (*A. flavicollis*) inhabited the wooded and shrubby habitats.

Patterns in population dynamics differed among species. The striped field mouse exhibited increasing abundance levels during spring and declined in summer, whereas the yellow necked mouse peaked in summer, remained high in autumn and declined to a trough in winter (Fig. 1).

Hantavirus detection and investigated individual and population factors associated with hantavirus transmission

The overall DOBV prevalence was 7.7% in *Apodemus flavicollis* and 1.43 % in *Apodemus agrarius*. Univariate analyses showed that yellow-necked mice were significantly more likely to have been PCR positive than field mice (OR 8.15, 95% confidence interval [CI] 3.08 to 21.59, p<0.001). The male rodents were significantly more likely to be PCR positive for DOBV Hantavirus (OR 5.49, CI 1.62 to 18.54, p=0.006) than the females. Reproductively active animals and senile individuals were also significantly and positively associated with the likelihood that rodents were PCR positive (OR 4.9, CI 1.54 to15.86, p=0.007; OR 8.84, CI 2.74 to 28.47, p<0.001) than the non active, sub adult animals respectively. In the most parsimonious multivariable model, yellow-necked mice remained significantly more likely to be PCR positive and rodents were significantly more likely to be PCR positive if they were reproductively active, male and overwintered individuals. The peak of the population phase seems to also be an important population demographic factor for the species *A. flavicollis*, with a 20.7-fold higher risk than the decrease population phase (p< 0.001). Because of insufficient data for DOBV PCR positive individuals of *A. agrarius*, no significant results for the population phase were obtained.

Discussion

This study provides evidence that the species A. *flavicollis* is a dominant reservoir host species for DOBV in the endemoepidemic process in Southern Bulgaria. This species is the main reservoir of the infective agent also in other countries in the Balkans (Petricevic *et al.* 1989; Kuzman *et al.* 1997).

Our results have confirmed and refined con-

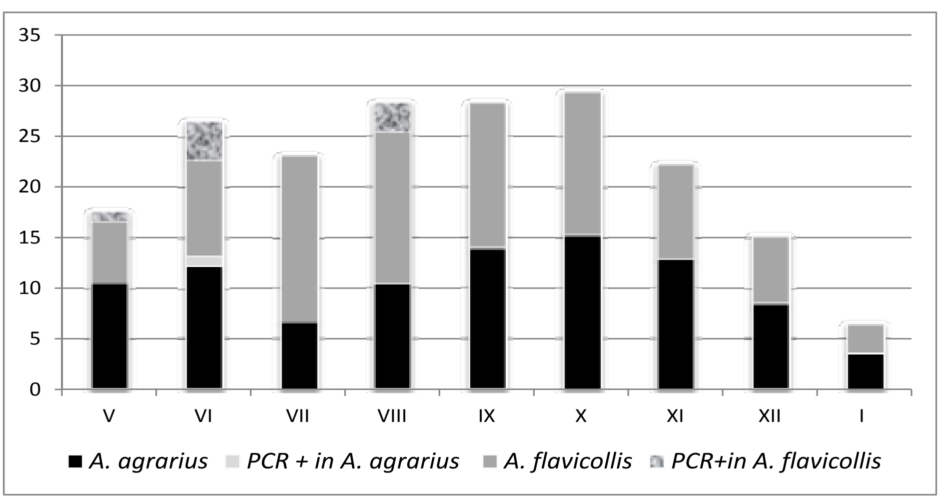

Fig. 1. Captured and PCR positive for DOBV individuals of yellow-necked and field mice

Table 1. Relationships between individual variables and PCR results of rodent samples in the most parsimonious, minimal multivariable logistic regression model

Variable	Coefficient (SE)	Z	p value	Odds ratio	95% CI
A. flavicollis vs ***A. agrarius***	1.894 (0.468)	4.047	<0.001	6.65	2.66 to 16.64
Male vs. **female**	1.239 (0.556)	2.228	0.03	3.45	1.16 to 10.28
Repr. active vs. **non active**	2.369 (0.735)	3.224	0.001	10.69	2.53 to 45.09
Senex vs. **subadult**	2.917 (0.629)	3.567	0.007	4.9	1.54 to15.86

clusions of several studies demonstrating the association of hantavirus infections with gender, age and reproductive status. Adult males are often the most frequently infected animals in a population during the breeding season (Klein 2005; CHARLS *et al*. 2007). This has been observed for PUUV infections in bank voles from France (Sauvage 2004; Deter *et al.* 2007), the Ural Mountains (Bernshtein *et al.* 1999) and Sweden (Olsson *et al.* 2002). The sex-related bias in the odds of infection is likely caused by differences in social behavior between males and females (Mills *et al.* 1999; Bernshtein *et al.* 1999; Olsson *et al.* 2002). Aggressive behavior, such as biting, between males has been suggested as a means of hantavirus transmission in other studies (Glass *et al*. 1988). The males of *Apodemus* spp. do not defend territories but usually have overlapping home ranges in their competition for mating. During the reproductive season, breeding males travel over more female territories and frequently visit ''excretory points'' and shelters of other animals, increasing their risk of acquiring infection. More intensive mobility and dispersal activity of young maturing males compared to reproducing females was proposed as an explanation by Bernshtein (1999). Young, reproductively inactive mice have probably not dispersed yet, whereas territories inhabited by adults are continually visited by dispersers. Females, however, compete for food and defend territories (Olsson *et al.* 2002). When our data are compared to those from previously published studies regarding hantavirus infections, the degree of males being PCR DOBV positive among adult rodent hosts appears to be greater than in some other host species e.g., bank vole for PUMALA (Olsson *et al*. 2002; Tersago *et al.* 2011) and common vole for TULV-seroprevalence (Deter *et al.* 2007). Careful comparative analyses will be required to determine whether these differences are because of site differences, or because of differences in social structure and behavior among hosts (Mills *et al*. 1999).

The other important factor with high influence on the probability of being PCR DOBV positive was the mice age at capture. Age is an important epidemiologic parameter because chance of exposure to horizontally transmitted pathogens generally increases with age (Olsson *et al.* 2005). The obtained results show that horizontal infection is crucial in DOBV transmission among *Apodemus* spp. Evidence of horizontal infection has previously been documented for Hantavirus in rats, (Glass *et al.* 1988) and bank voles (Olsson *et al.* 2005). The influence of age indicates that aggressive contacts and fighting among males are probably not the only mechanism of infection. Other mechanisms such as naso-nasal and naso-anal contacts, mutual grooming, aerosol, or venereal transmission also play a role in the spread of the virus among hosts and therefore longevity in and near the trapping site may be also an important factor (Calisher *et al.* 2009).

The importance of reproductive activity in relation to individual Hantavirus infection risk in the reservoir host has been mentioned several times and in different hantavirus– host systems (Glass *et al.* 1989, Bernshtein *et al*. 1999, Mills 2005). These observations could be due to several mechanisms. Juveniles can be temporarily protected by maternal antibodies transferred from their DOBV-infected mothers. Consequently, DOBV transmission toward young mice will likely decrease in the subsequent breeding period (Kallio *et al*. 2006, Tersago *et al.* 2011). These young inactive mice were likely no longer protected by maternal antibodies and their contacts related to reproductive behavior became increasingly important. The reproductively active mice remained infected throughout their lifetime. Territoriality and aggression, related to reproductive behavior could lead to higher infection risk in populations of yellow-necked and striped field mice.

On the basis of the obtained results and those in the existing literature, overwintered reproductively active males of yellow-necked mice appear critical to the success of DOBV circulation and persistence within host populations in Southern Bulgaria.

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