Associations between MHC genes and Puumala virus infection in *Myodes glareolus* are detected in wild populations, but not from experimental infection data

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We analysed the influence of MHC class II *Dqa* and *Drb* genes on Puumala virus (PUUV) infection in bank voles (*Myodes glareolus*). We considered voles sampled in five European localities or derived from a previous experiment that showed variable infection success of PUUV. The genetic variation observed in the *Dqa* and *Drb* genes was assessed by using single-strand conformation polymorphism and pyrosequencing methods, respectively. Patterns were compared with those obtained from 13 microsatellites. We revealed significant genetic differentiation between PUUV-seronegative and -seropositive bank voles sampled in wild populations, at the *Drb* gene only. The absence of genetic differentiation observed at neutral microsatellites confirmed the important role of selective pressures in shaping these *Drb* patterns. Also, we found no significant associations between infection success and MHC alleles among laboratory-colonized bank voles, which is explained by a loss of genetic variability that occurred during the captivity of these voles.

Puumala virus (PUUV) is a rodent-borne, enveloped RNA virus belonging to the genus *Hantavirus* (family *Bunyaviridae*). In humans, it is responsible for a mild haemorrhagic fever with renal syndrome (Lundkvist & Niklasson, 1992). Symptoms vary between human patients (Lahdevirta, 1971; Mustonen et al., 1994). Genetic studies have revealed the influence of HLA haplotypes on the severity of PUUV infection in humans (Mustonen et al., 1996; Plyusnin et al., 1997).

The bank vole, *Myodes glareolus*, is the reservoir species of PUUV in Europe. PUUV infection in bank voles is chronic (Hardestam et al., 2008) and asymptomatic (Meyer & Schmaljohn, 2000). Rodents were therefore considered to be healthy carriers of the virus (Yanagihara et al., 1985). However, recent studies have revealed that PUUV infection...
reduced the winter survival of infected individuals (Kallio et al., 2007). Also, field surveys and experiments indicate that some variation exists among bank voles in their probability of being infected with PUUV (e.g. Deter et al., 2008a; Olsson et al., 2002). Experimental infections have also shown that infectivity varies among bank voles (Hardestam et al., 2008; Kallio et al., 2006). This variability could be related to immunogenetic background. First, a large number of genes that encode proinflammatory, antiviral, MHC, IgG and T-cell marker proteins are upregulated in female compared with male Norway rats following infection with Seoul virus (SEOV) (Klein et al., 2004). This upregulation leads to an increased ability of females to control SEOV replication, and we can assume that it could also prevent infection in the first place. Second, an epidemiological survey has revealed associations between MHC alleles and PUUV patterns of infection in wild bank vole populations (Deter et al., 2008b). However, in that study, uninfected bank voles might never have been exposed to PUUV, and therefore it is not certain that these alleles influence susceptibility to PUUV directly. Altogether, these preliminary observations raise questions about the existence of genetic factors underlying the variability of bank vole susceptibility to PUUV infection. In this context, MHC genes deserve particular attention, as their polymorphism seems to influence the ability of humans and rodents to control hantavirus replication (Klein et al., 2004; Mäkelä et al., 2002; Plyusnin et al., 1997; Terajima et al., 2004). They could mediate differences in efficiency to prevent hantavirus infection.

Our study focused on two MHC class II genes, Dqa and Drb. First, we looked for associations between MHC haplotypes and PUUV infection in wild populations. We compared five localities that differed in their level of PUUV prevalence. Three corresponded to areas of high PUUV prevalence in Fennoscandia [Ilmajoki (Finland), Konnevesi (Finland) and Västerbotten (Sweden)] and two others corresponded to lower PUUV prevalence [Ardennes: Elan (France) and Beaumont (Belgium)] (Vapalahti et al., 2003). Individual blood samples were screened for anti-PUUV immunoglobulins using an immunofluorescence antibody test as described by Kallio-Kokko et al. (2006). We included about 30 individuals per locality, with about half of PUUV-seropositive voles. Details are provided in Supplementary Table S1, available in JGV Online. For each vole, we stored a piece of ear or toe in 96 % ethanol for genetic analyses. In areas of high prevalence, we expected to find a strong selective pressure imposed on bank vole by PUUV. Therefore, MHC haplotypes should be associated strongly with PUUV susceptibility. This would result in strong genetic differentiation at MHC genes between PUUV-seropositive and -seronegative voles. Finally, we also expected differences in PUUV lineages to drive local adaptation processes (e.g. Asikainen et al., 2000; Johansson et al., 2008). In consequence, MHC haplotypes associated with PUUV infection should depend on the localities studied. Second, we used the bank vole material from the experiment of Kallio et al. (2006), where naive recipient voles were exposed to beddings previously contaminated by vole donors inoculated with PUUV strain Kazan [see details given by Kallio et al. (2006) and in Supplementary Fig. S1, available in JGV Online]. Infection success, as determined by RT-PCR, was highly variable among recipient voles. We aimed to assess whether this variability could result from different MHC haplotypes in recipient bank voles. We included the 101 recipient voles of the study (referred to as the S1 dataset), or we selected a subsample (S2) of 62 voles corresponding to those known to have been exposed to PUUV-contaminated beddings. Indeed, even if some recipient voles were uninfected, their bedding was shown to infect subsequent recipient voles further exposed to it. A piece of lung stored in 96 % ethanol was used for genetic analyses.

Genomic DNA was extracted by using a DNeasy 96 kit (Qiagen) according to the manufacturer’s instructions. We amplified the complete exon 2 of the MHC class II gene Dqa (Dqa-exon2) following the protocol described by Bryja et al. (2006), using fluorescein-labelled primers (forward primer labelled by 6-FAM and reverse by HEX) and 35 cycles of denaturation/annealing/extension. Single-strand conformation polymorphism (SSCP) analyses of PCR products were then performed by capillary electrophoresis (CE) on a MegaBACE 1000 DNA-analysis system (Amersham Biosciences) following Bryja et al. (2005). The electropherograms were aligned and analysed with the software MegaBACE Genetic Profiler 1.5 (Amersham Biosciences). Two alleles (Clgl-Dqa*08 and Clgl-Dqa*35; GenBank accession nos EU371603 and EU371614, respectively) differed only by 1 bp and exhibited indistinguishable CE-SSCP patterns. We thus applied an RFLP test based on HphI and PdmI enzymic restrictions to discriminate these alleles, even in the presence of other alleles (P.-J. G. Malé, J. F. Martin, M. Galan, J. Bryja, V. Deffontaine, J.-F. Cosson, J. Michaux & N. Charbonnel, unpublished data). Note that, for technical reasons, PUUV-seronegative vole samples from Västerbotten have unfortunately not been genotyped at the Dqa-exon2 gene. We amplified exon 2 of the MHC class II gene Drb (Drb-exon2) using the 454 GS FLX (Roche) platform. We used the primers JS1 (forward; 5′-GAGTGTCATTTCTACAA-CGGGAGG-3′) and JS2 (reverse; 5′-GATCCCGTAGTTG-TGTTGGC-3′) (Schad et al., 2005). The combination of tags in the reverse and forward primers formed unique barcode identifiers and allowed the reassembly of each of the thousands of sequences obtained from the 434 pyrosequencing runs to a unique individual. A stepwise bioinformatic procedure was developed to provide individual genotypes (Galan et al., 2010). The Drb-exon2 sequences were aligned in the BioEdit sequence alignment editor (Hall, 1999) using CLUSTAL_X multiple alignment. Nomenclature of the bank vole sequences follows Klein et al. (1990). The sequences are available in GenBank under accession numbers HM347503–HM347507 and HM107849–HM107870. Human Homo sapiens (GenBank accession nos
M17236, AM109973), sheep *Ovis aries* (M33304, AY230000) and pig *Sus scrofa* (M29938, NM_001113695) sequences were used as outgroups in phylogenetic analyses. MEGA4 software (Tamura et al., 2007) was employed to construct a phylogenetic tree of the *Drb*-exon2 gene using the neighbour-joining algorithm and Kimura two-parameter distance model. A bootstrap analysis (1000 replicates) was performed to determine the reliability of the branching. We confirmed that *Drb*-exon2 was duplicated in *M. glareolus* (Axtner & Sommer, 2007), although phylogenetic reconstructions did not enable us to assign all alleles to one or another copy of the gene (Supplementary Fig. S2, available in JGV Online). However, we identified different alleles as belonging to cluster I (Axtner & Sommer, 2007).

In natural populations, MHC genetic differentiation between PUUV-seronegative and -seropositive bank voles could result from neutral processes, such as strong familial structure, or from selective ones. To discriminate the influence of these forces, we compared the genetic patterns observed at the *Dqa*-exon2 and *Drb*-exon2 genes with those obtained from microsatellites, which are supposed to be neutral (Luikart et al., 2003). Therefore, we genotyped the bank vole samples corresponding to natural populations and the transmission experiment at 17 microsatellite loci using the primer sets developed by Rikalainen et al. (2008), except for primers Cg1F11, Cg17C9, Cg2F2, Cg4F9 and Cg1G12, which have been redesigned. Genotyping was carried out using an ABI 3130 automated DNA sequencer.

![Discriminant analyses performed on MHC class II gene polymorphism with regard to PUUV serological status and locality. (a, b) Dqa and Drb exon 2 gene results, respectively; (c) discriminant scores for the Drb-exon2 gene. B, E, I, K and V correspond to localities sampled [Beaumont (Ardennes), Elan (Ardennes), Ilmajoki (Finland), Konnevesi (Finland) and Västerbotten (Sweden), respectively]. – and + respectively correspond to PUUV-seronegative and -seropositive bank voles. Circles represent the barycentre of each PUUV-locality group.](http://vir.sgmjournals.org/)

**Fig. 1.** Discriminant analyses performed on MHC class II gene polymorphism with regard to PUUV serological status and locality. (a, b) Dqa and Drb exon 2 gene results, respectively; (c) discriminant scores for the Drb-exon2 gene. B, E, I, K and V correspond to localities sampled [Beaumont (Ardennes), Elan (Ardennes), Ilmajoki (Finland), Konnevesi (Finland) and Västerbotten (Sweden), respectively]. – and + respectively correspond to PUUV-seronegative and -seropositive bank voles. Circles represent the barycentre of each PUUV-locality group.
(Applied Biosystems) with labelling of the 5′-end forward primer with fluorescent dye and separation into two sets (set 1: FAM: Cg15F7, Cg10A11, Cg1F11; HEX: Cg2F2, Cg13G2, Cg16E4; NED: Cg13F9, Cg3A8; set 2: FAM: Cg16A3, Cg13C12, Cg17C9; HEX: Cg4F9, Cg3F12, Cg14E1; NED: Cg6G11, Cg2C5, Cg12A7). Four microsatellites (Cg3F12, Cg2F2, Cg17C9 and Cg13F9) showed null alleles and were removed. The mean allelic richness was estimated at these microsatellites for each population by using the rarefaction procedure implemented in FSTAT 2.9.3.2 (Goudet, 2001).

We first performed multivariate analyses using ADE-4 software (Thioulouse et al., 1997) to determine the MHC alleles that best discriminated among seropositive/seronegative groups within the five localities sampled and for the experimental datasets. Discriminant function analysis was used to maximize the variance between designated groups (serology*locality) while keeping the intra-group variance constant (Jombart et al., 2009). The significance of the ratio of these two values was estimated by using 10,000 permutation tests. For each allele, we estimated the relative risk (RR) following Haldane (1956) and we tested the association with vole serological status by using Fisher’s exact tests and Bonferroni sequential corrections. Next, we tested the conformity to Hardy–Weinberg equilibrium for each microsatellite locus and locality using the Markov chain methods implemented in GENEPOP v. 3.3 (Raymond & Rousset, 1995). We looked for signatures of selection by using AMOVA in ARLEQUIN v. 3.0 software (Excoffier et al., 2005). We estimated the proportion of variance and F-statistics ($\Phi_{CT}$, $\Phi_{SC}$, $\Phi_{ST}$) at three hierarchical levels of subdivision: among the five localities sampled, among the serological groups within each locality and within each serological group. The significance of these parameters was evaluated by using 10,000 permutations. AMOVAs were performed independently for microsatellites on one hand and for Drb cluster I on the other hand. The detection of significantly contrasting patterns of genetic differentiation at Drb with regard to microsatellites would reveal the action of selection on Drb (e.g. Spurgin & Richardson, 2010).

We performed discriminant analyses on the wild population dataset, considering 124 bank voles (57 seropositive) and seven alleles (three others had frequencies <5%) for the Dqa gene, and considering 130 voles (68 seropositive) and 11 alleles (22 others had frequencies <5%) for the Drb gene. Both analyses revealed significant differences among serology/locality groups ($Dqa: P<10^{-4}$; $Drb: P<10^{-4}$). Regarding the Dqa gene, the two first discriminant factors, which represented respectively 47.7 and 24.6 % of the total inertia, only revealed geographical oppositions between the Finnish and the Ardennes populations, whatever the serological status of individuals (Fig. 1a). Again, this result did not support the influence of the Dqa-exon2 gene on the probability of bank voles to be infected with PUUV. The Drb gene gave a different pattern, as the first axis of the discriminant analysis (29.7 % of the total inertia) separated seropositive bank voles of the Ardennes well from those of Ilmajoki (Fig. 1b). This was explained by the allele Mygl-Drb*03, which was not found in the Ardennes populations and exhibited a high RR in Ilmajoki (RR=3.73; Fisher’s exact test, $P=0.069$), and by the allele Mygl-Drb*10, which was not found in Ilmajoki but exhibited a high RR in the Ardennes (Beaumont: RR=6.44; Fisher’s exact test, $P=0.098$; Elan: RR=2.39; Fisher’s exact test, $P=0.232$; Fig. 1c). The second axis (25.2 % of the total inertia) also discriminated seropositive bank voles sampled in Konnevesi and Västerbotten from all voles of the Ardennes. The allele Mygl-Drb*93 mostly explained this pattern (Fig. 1c), as it was associated with high RR in Västerbotten (RR=2.95; Fisher’s exact test, $P=0.047$) and Konnevesi (RR=1.8; Fisher’s exact test, $P=0.500$), whereas it was not found in the Ardennes. As expected, PUUV-seronegative and -seropositive voles were discriminated more significantly in Finland and Sweden than in the Ardennes (Fig. 1b). No significant molecular variance was observed between serological groups on the basis of microsatellites ($\Phi_{SC} = 0.00124; P=0.374$; Table 1), whereas it was significant when considering Drb cluster I ($\Phi_{SC} = 0.03998; P=0.001$).

No polymorphism could be detected at the Dqa-exon2 gene of the 101 bank voles from the experimental datasets (S1, S2). Also, we found seven Drb alleles. The discriminant analyses performed on S1 and S2 were not significant (S1: $P=0.741$, S2: $P=0.675$). Fisher’s exact tests revealed no significant differences in infection status distribution.

### Table 1. Results of AMOVA based on 13 microsatellites or on cluster I of Drb-exon2 for M. glareolus sampled in five localities

<table>
<thead>
<tr>
<th></th>
<th>Variation (%)</th>
<th>Statistics</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Based on 13 microsatellites</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among localities</td>
<td>6.99</td>
<td>$\Phi_{CT} = 0.06985$</td>
<td>$&lt;10^{-6}$</td>
</tr>
<tr>
<td>Among serogroup within localities</td>
<td>0.11</td>
<td>$\Phi_{SC} = 0.00124$</td>
<td>0.374</td>
</tr>
<tr>
<td>Among individuals within serogroups</td>
<td>92.90</td>
<td>$\Phi_{ST} = 0.07100$</td>
<td>$&lt;10^{-6}$</td>
</tr>
<tr>
<td>Based on cluster I of Drb-exon2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among localities</td>
<td>5.95</td>
<td>$\Phi_{CT} = 0.05946$</td>
<td>0.002</td>
</tr>
<tr>
<td>Among serogroup within localities</td>
<td>3.76</td>
<td>$\Phi_{SC} = 0.03998$</td>
<td>0.001</td>
</tr>
<tr>
<td>Among individuals within serogroups</td>
<td>90.29</td>
<td>$\Phi_{ST} = 0.09706$</td>
<td>$&lt;10^{-6}$</td>
</tr>
</tbody>
</table>
The whole dataset (S1) or only individuals known to have been exposed to infected bedding (S2) were considered.

<table>
<thead>
<tr>
<th>Dataset</th>
<th>Test</th>
<th>Mygl-Drb*08</th>
<th>Mygl-Drb*115</th>
<th>Mygl-Drb*116</th>
<th>Mygl-Drb*42</th>
<th>Mygl-Drb*117</th>
<th>Mygl-Drb*119</th>
<th>Mygl-Drb*118</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>Fisher’s exact test</td>
<td>0.495</td>
<td>0.127</td>
<td>0.571</td>
<td>0.454</td>
<td>0.086</td>
<td>0.609</td>
<td>0.316</td>
</tr>
<tr>
<td>RR</td>
<td></td>
<td>1.113</td>
<td>0.566</td>
<td>0.993</td>
<td>0.866</td>
<td>2.876</td>
<td>0.961</td>
<td>6.619</td>
</tr>
<tr>
<td>S2</td>
<td>Fisher’s exact test</td>
<td>0.557</td>
<td>0.227</td>
<td>0.462</td>
<td>0.567</td>
<td>0.062</td>
<td>0.524</td>
<td>0.516</td>
</tr>
<tr>
<td>RR</td>
<td></td>
<td>0.919</td>
<td>0.608</td>
<td>1.210</td>
<td>1.056</td>
<td>4.824</td>
<td>1.612</td>
<td>2.904</td>
</tr>
</tbody>
</table>

whatever the Drb allele and the dataset considered (Table 2), although the relative risks associated with Mygl-Drb*117 were high (S1: RR=2.87, P=0.008; S2: RR=4.82, P=0.062). These results indicated that the two MHC class II alleles did not play a major role in the differences in susceptibility to infection observed in this experiment. However, it should be noticed that this laboratory stock of bank voles had been maintained in captivity for 20 years. Therefore, inbreeding and loss of genetic variability could have prevented the detection of genetic associations. This was confirmed by the low allelic richness observed at microsatellites among these experimental samples. A mean of 2.9 alleles was observed, which is far lower than that detected in wild populations (between 8.6 and 12.2 alleles; see Supplementary Table S1). Furthermore, Kallio et al. (2006) showed that other individual factors (which might be important in nature), such as sex, weight and sexual maturity, did not explain differences in susceptibility to PUUV within this experiment. Sironen et al. (2008) suggested that variability in PUUV S segment sequences could have mediated the variability in infection success observed in this experiment.

This immunogenetic study revealed clear associations between some Drb-exon2 alleles and PUUV serological status in wild bank vole populations. This genetic differentiation between PUUV-seronegative and -seropositive bank voles within localities was not explained by neutral processes, as reflected by the absence of differenti-ation detected at microsatellites. It is likely that it was mediated by selective pressures acting on the Drb-exon2 gene. As expected, the selective pressure mediated by PUUV on the Drb gene seemed stronger in Finland and Sweden than in the Ardennes. We therefore concluded that the associations between Drb-exon2 alleles and PUUV serological status observed in the wild could be driven directly by PUUV itself. However, we could not exclude the possibility that other parasites present in the environment had mediated the observed patterns. The experimental infection reported no obvious direct role for the Drb-exon2 gene in PUUV infection success. The low level of genetic diversity in laboratory voles did not allow testing of the hypothesis of associations between Drb-exon2 alleles and susceptibility to PUUV. This last result pointed out the necessity to perform future experimental infections using wild trapped voles with consistent genetic diversity. Finally, our study confirmed that Drb-exon2 alleles associated with PUUV serological status differed among localities. Understanding the impact of such local adaptation processes on PUUV infectivity, with regard to M. glareolus population genetics or phylogeography, now deserves further investigation.

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