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Association between the DQA MHC class II gene and Puumala virus infection in *Myodes glareolus*, the bank vole

Julie Deter [a,*], Josef Bryja [b], Yannick Chaval [a], Maxime Galan [a], Heikki Henttonen [c], Juha Laakkonen [c,d], Liina Voutilainen [c], Olli Vapalahti [d], Antti Vaheri [d], Alexis Ribas Salvador [e], Serge Morand [f], Jean-François Cosson [a], Nathalie Charbonnel [a]

[a] *Centre de Biologie et de Gestion et des Populations (CBGP), Département INRA-EFPA 1062, Campus International de Baillarguet, CS 30016, 34988 Montferrier sur lez, France*
[b] *Department of Population Biology, Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, 67502 Studenec 122, Czech Republic*
[c] *Vantaa Research Unit, Finnish Forest Research Institute, PO Box 18, FIN-01301 Vantaa, Finland*
[d] *Department of Virology, Haartman Institute, PO Box 21, FIN-00014 University of Helsinki, Finland*
[e] *Laboratori de Parasitologia, Departament de Microbiologia i Parasitologia Sanitàries, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII s/n, E-08028 Barcelona, Spain*
[f] *Institut des Sciences de l'Evolution, CNRS, CC064, Université Montpellier 2, 34095, Montpellier Cedex 05, France*

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Abstract

Puumala virus (PUUV) is a hantavirus specifically harboured by the bank vole, *Myodes* (earlier *Clethrionomys*) *glareolus*. It causes a mild form of hemorrhagic fever with renal syndrome (HFRS) in humans, called *Nephropathia epidemica* (NE). The clinical severity of NE is variable among patients and depends on their major histocompatibility complex (MHC) genetic background. In this study we investigated the potential role of class II MHC gene polymorphism in the susceptibility/resistance to PUUV in the wild reservoir *M. glareolus*. We performed an association study between the exon 2 of the DQA gene and PUUV antibodies considering a natural population of bank voles. Because immune gene polymorphism is likely to be driven by multiple parasites in the wild, we also screened bank voles for other potential viral and parasitic infections. We used multivariate analyses to explore *DQA* polymorphism/PUUV associations while considering the potential antagonist and/or synergistic effects of the whole parasite community. Our study suggests links between class II MHC characteristics and viral infections including PUUV and Cowpox virus. Several alleles are likely to be involved in the susceptibility or in the resistance of bank voles to these infections. Alternatively, heterozygosity does not seem to be associated with PUUV or any other parasite infections. This result thus provides no evidence in favour of the hypothesis of selection through overdominance. Finally this multivariate approach reveals a strong antagonism between ectoparasitic mites and PUUV, suggesting direct or indirect immunogenetic links between infections by these parasites. Other datasets are now required to confirm these results and to test whether the associations vary in space and/or time.

Keywords: *Myodes* (*Clethrionomys*) *glareolus*; Hantavirus; SSCP; Coinertia; Helminths; Mites; Cowpox virus

1. Introduction

1.1. Immunogenetics and zoonoses

Polymorphism at immune genes may be involved in specific recognition of pathogens as variation allows the recognition of spectra of epitopes (Doherty and Zinkernagel, 1975). In this context, one of the leading goals of immunogenetics, i.e. the analysis of genetic polymorphisms in specific recognition and immune regulation, has been to understand the genetic basis of susceptibility to complex diseases (Geraghty, 2002). The profound influence of the host genetics on resistance to infections has been established in numerous studies, which mainly concerned human infections such as malaria, HIV and hepatitis (review in Cooke and Hill, 2001; Hill, 2001). In wild

* Corresponding author. Tel.: +33 4 99 62 33 46; fax: +33 4 99 62 33 45.
E-mail address: deter@supagro.inra.fr (J. Deter).

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animal populations, immunogenetics provides key insight into the relative influence of genetic variation and environmental factors on host-pathogen interactions. A number of zoonoses, i.e. infections transmitted from animals to humans (Taylor et al., 2001), has (re-)emerged during the past 15 years (Kallio-Kokko et al., 2005). Therefore, an important application of immunogenetics concerns the assessment of emergent or re-emergent disease risks in natural populations. (i) The study of immune genes may explain why hosts differ in their susceptibility to different parasites. The reasons why hosts differ in their susceptibility/resistance to different parasites could rely on the degree of matching between immune genes and parasite antigens. Immunogenetics may thus contribute to the identification of unknown zoonotic agents, which is essential for understanding zoonoses epidemiology (Mills and Childs, 1998). (ii) Natural population studies of immune gene polymorphism may provide key insight into the factors determining the appearance, spread and distribution of resistance/immuno-modulating alleles within populations and across geographical landscapes. Such information is essential to study the spatial and temporal variations in disease risk or incidence.

1.2. Puumala virus and hemorrhagic fever with renal syndrome

Hantaviruses are one of the three main emerging infectious agents in Europe (Kallio-Kokko et al., 2005). They are specific rodent-borne viruses belonging to the Bunyaviridae family, and are transmitted to humans primarily from aerosols of rodent excreta. Puumala virus (PUUV) is a hantavirus, which causes a mild form of haemorrhagic fever with renal syndrome (HFRS) in humans, called *Nephropathia epidemica* (NE). The mortality rate ranges between 0.1 and 0.4% (Vapalahti et al., 2003). Infections are frequent in Northern Europe, European Russia and parts of Central Western Europe (Vapalahti et al., 2003), with about 6000 cases reported per year. In France, it has been classified as an important emerging disease by the Institut de Veille Sanitaire (INVS, Capek, 2006).

The specific reservoir host of PUUV is the bank vole *Myodes* (earlier *Clethrionomys*) *glareolus*. In this rodent, PUUV produces a chronic, long-lasting infection. Host and virus have geographical ranges that do not completely overlap. Many parts of Europe, such as the Mediterranean peninsulas and Britain remain blank of PUUV infections although *M. glareolus* is present. Two recent studies provided arguments in favour of selection acting on bank voles and mediated by PUUV. First, Kallio et al. (2006a,b) observed that PUUV infection could affect bank vole fecundity (earlier breeding). Second, experimental infections of *M. glareolus* laboratory colonies with PUUV revealed that some bank voles could not be successfully infected. Neither antibodies, nor PUUV RNA could be detected in these individuals reared in PUUV-contaminated beddings, providing evidence of variability in vole susceptibility to PUUV. This variability was not significantly explained by age, sex, maturation status or individual weight (Kallio et al., 2006a,b). This suggests that immunogenetic factors could be involved not only in the disease progression (Klein et al., 2004a) but also in controlling infection itself. The absence of HFRS in some parts of *M. glareolus* geographic distribution could therefore be partly explained by selected vole immunogenetic factors associated with resistance to PUUV infections.

1.3. Immunogenetic factors associated with Puumala virus infections

Association studies have provided evidence for the role of immunogenetics in the course of hantavirus infections in rodents. Using the hantavirus *Seoul* and the rat *Rattus norvegicus*, Klein et al. (2004a,b) characterized immunologic pathways that differ between males and females in response to infection. They showed that most of the 192 identified immune-related genes that encode for these immune responses were differentially expressed in infected females compared with infected males. Several MHC genes were associated with these sex differences in gene expression. Alternatively, the considerable variability in the clinical severity of NE among humans depends on MHC genes (Mustonen et al., 1996, 1998, 2004; Plyusnin et al., 1997). The HLA-B227 haplotype is associated with a mild course of NE, whereas the extended haplotypes HLA-B8-DR3 and HLA-DRB1-0301 are associated with severe clinical courses. The complete MHC class II region has been sequenced in rats (Günther and Walter, 2001; Hurt et al., 2004) and mice (Blake et al., 2003). It has a similar regional organization as the human MHC, and orthologous relationships exist between class II regions in all mammals (Takahata and Nei, 1990). Moreover, in laboratory mouse and rats, the DQA gene (called RT1.B in rats, H2-A in mice) is tightly linked to DRB genes (Blake et al., 2003; Hurt et al., 2004). As DRB is at least quadriduplicated in bank voles (Axtner and Sommer, 2007), what makes genotyping difficult to perform, DQA was a relevant candidate to look for associations between MHC class II genes and PUUV infection.

In this study we investigate the role of the class II MHC *DQA* gene polymorphism on susceptibility/resistance to PUUV by the mean of an association study conducted in a natural population of *Myodes glareolus*, the specific reservoir of this hantavirus. Vole sampling was performed within the second most important French endemic area of HFRS (Jura, Franche Comté), which has had an unusually high number of human cases in 2005 (230 in France, 38 in the Jura, INVS).

2. Materials and methods

2.1. Trapping design

The main study area is located around Mignovillard (46°8′N, 6°13′E and elevation 850 m) in Jura, France. It consists of a 4-km^2 site, half of which is composed of wooded meadows and the other half of forests (man-made spruce or semi-natural forests). Bank voles were sampled in September 2004, July and September 2005 using French Agricultural Research Institute (INRA) live traps, which were fitted out with dormitory boxes,

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baited with hay and a piece of apple. Twenty 100-m trap-lines composed of 34 traps placed at 3-m intervals were equally distributed in open (meadows and hedges, nine lines) and wooded (five lines in forests and six lines in meadows in border of forest) areas. For each trapping session, these lines were checked twice a day during three consecutive nights.

2.2. Infection screening

Once trapped, rodents were killed by cervical dislocation as recommended by Mills et al. (1995). For each individual, blood samples were taken from the heart or the thoracic cavity on a 1-cm^2 Whatman® blotting paper. In the laboratory, each piece of 1-cm^2 paper with dried blood was placed in 1 ml phosphate-buffered saline. These diluted blood samples were screened for IgG antibodies to Puumala virus (PUUV) using immunofluorescence assays (IFA) as described in Lundkvist et al. (1991) (see also Klingström et al., 2004; Kallio-Kokko et al., 2005). As PUUV produces a long-lasting, chronic infection in bank voles, the detection of antibodies for PUUV is strongly related with the presence of the virus except for very young voles because of possible maternal antibodies.

Because immune gene polymorphism is likely to be driven by multiple parasites in the wild (Chantrey et al., 1999; Wegner et al., 2003), we also screened bank voles for other potential viral and parasitic infections. *M. glareolus* is considered as one of the main reservoir of Cowpox virus (CPXV, Chantrey et al., 1999). Moreover, recent serological surveys have revealed the potential role of *M. glareolus* as a reservoir of the Lymphocytic Choriomeningitis virus (LCMV) (Kallio-Kokko et al., 2006). We thus used vole blood samples to screen for IgG antibodies against CPXV and LCMV by IFA as described in (Kallio-Kokko et al., 2005, 2006; Laakkonen et al., 2006). On the field, the presence of ectoparasitic mites was visually inspected and noted. Whole fresh brains were squashed and checked microscopically for the presence of *Frenkelia* cysts (Coccidia, Apicomplexa: Sarcocystidae). Finally, we looked for the presence of larval cestodes in the liver and in the body cavity of voles. The digestive tracts (stomach, intestines) were stored in 95% ethanol before being dissected in the laboratory. All the helminths detected were carefully counted under the microscope and then identified in Barcelona.

2.3. MHC class II gene genotyping

Genomic DNA was extracted from toe tissues by Puregene DNA purification kit® (Gentra Systems) according to manufacturer's instructions and finally eluted in 400-μl of water. We amplified the complete exon 2 of *DQA* (called *RT1.Ba* in rat) homologue of the MHC class II as described in Bryja et al. (2005, 2006). A single PCR (PCR1 in Bryja et al. (2006)) was performed on an Epgradient® thermocycler (Eppendorf) as it allowed the amplification of all bank vole alleles. Capillary electophoreses-single strand conformation polymorphism (CE-SSCP) were performed on a MegaBACE 1000 DNA Analysis system® (Amersham Biosciences) as described in Bryja et al. (2006). All alleles detected following the CE-SSCP analyses were cloned and sequenced using a minimal subset of animals exhibiting the diverse SSCP patterns observed. Briefly, the *DQA* gene was amplified as described above, but using non-labelled primers. The PCR products were cloned and purified by the QIAquick PCR purification kit® (Promega) as described in Bryja et al. (2006). Eight clones were subsequently isolated for each individual selected. The plasmid DNA was extracted using QIAprep Spin Miniprep kit® (Qiagen). The *DQA* insert was sequenced using DYEnamic ET terminator cycle sequencing kit® (Amersham Biosciences) and primer SP6. Sequences were performed on MegaBACE 1000 DNA analysis system® (Amersham Biosciences). They were edited and aligned using BioEdit Sequence Alignment Editor 7.0.5.2 (Hall, 1999). A new sequence variant was considered as a new allele when it met the criteria summarised by Kennedy et al. (2002). Paup version 4.0b10 for Microsoft Windows 95/NT was employed to construct a phylogenetic tree of the *DQA* amino acid sequences based on 247 bp of the exon 2 (base pairs no. 3-249 of the complete exon), applying the neighbour-joining (NJ) algorithm with Kimura's two-parameter distances for nucleotides, and the Poisson correction for amino acids. Bootstrap analyses (5000 replicates) were performed to determine the reliability of the branching. *R. norvegicus* (GenBank accession no. AJ554214) and *Mus musculus* (no. K01924) sequences were used as outgroups in the phylogenetic analyses.

2.4. Statistical analyses

Seroprevalence (percentage of seropositive animals) for each virus and prevalence (percentage of infected animals) for each parasite were calculated and 95% confidence intervals were estimated by bootstraps using the software Quantitative Parasitology 3.0 (Rozsa et al., 2000).

We explored the immunologic factors (*DQA* alleles and heterozygosis) potentially associated with PUUV infection status using multivariate analyses, which affords the integration of other viruses, ectoparasites, coccidia and helminths. This gave the opportunity to explore at the same time the antagonist and/or synergistic effects of other parasites on *DQA* polymorphism/PUUV associations. We performed a co-inertia analysis (ACO) between (i) a genetic matrix including the presence/absence of each *DQA* allele and the *DQA* heterozygosis for each individual, and (ii) a parasitological matrix including the abundance (when available) or presence of each parasite species and the presence of antibodies against each virus for each individual. The ACO links the independent multivariate analyses of the genetic matrix (correspondence analysis, COA) and of the parasitological matrix (normalised principal component analysis, PCA). It considers all types of variables and it is robust to correlations between the variables of a matrix (Dray et al., 2003). The ACO gives co-inertia axes that maximize the covariance between the row coordinates of the two matrices (Dray et al., 2003; Thioulouse et al., 2004). The genetic and parasitological results can be compared by superimposing both kinds of variables on the ACO factor map. Therefore, particular associations were visually detected

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using the distribution of genetic and parasitological variables on the F1 × F2 ACO factor map. When the two first axes explained a large part of the co-inertia, the variables located in a given direction relative to the origin were considered positively associated, whereas the variables located in opposite directions were considered antagonistic. Variables plotted near the origin could not be interpreted. The ACO was performed using ADE-4 software (Thioulouse et al., 1997). The global relationship between the two matrices was investigated by comparing the ACO previously estimated to the distribution of co-inertia estimated from 1000 permutations of the parasitological matrix rows. Considering PUUV, the relative risk (RR) associated with the alleles of interest detected using the ACO was estimated following Haldane (1956).

Besides revealing associations between immunogenetics and PUUV infection, this multivariate analysis allows to discriminate two mechanisms underlying selection mediated by pathogens/parasites and acting on *DQA* gene. First, under the hypothesis of frequency-dependent selection (Takahata and Nei, 1990), we expect positive or negative associations between the presence of specific allele and parasite species. This association could be interpreted in terms of resistance (negative association) or susceptibility (positive) alleles to parasite species. Second, under the hypothesis of heterozygosis advantage, i.e. overdominance (Doherty and Zinkernagel, 1975; Hughes and Nei, 1990), heterozygous voles are expected to be less parasitized than homozygous ones.

3. Results

The 98 bank voles, 53 males and 45 females, trapped between September 2004 and 2005 in Mignovillard were included in the analyses.

3.1. Infection screening

The IFA analyses revealed nine (9.2%) PUUV-seropositive bank voles. The sequencing of the hantavirus involved has been described elsewhere on the basis of the S-segment (Plyusnina et al., in press) and confirmed the presence of PUUV in bank voles. Fifteen (15%) of the bank voles were CPXV seropositive. No antibodies against LCMV were detected. Orange-coloured mites were found in the ear lobes of 48 bank voles. These were identified as larvae of the trombiculid *Neotrombicula inopinata* (Acari, Actinotrichida, Prostigmata). Sporocysts of *Frenkelia glareoli* (Apicomplexa: Sarcocystidae) were found in the brain of a single bank vole. Larvae of two cestodes, *Echinococcus multilocularis* (Cestoda: Cyclophyllidae) and *Taenia taeniaeformis* (Cestoda: Cyclophyllidae) were found encysted in the liver of respectively 11 and 29 voles. In gastro-intestinal tracts, we found the following helminths *Paranoplocephala gracilis* (Cestoda: Anoplocephalidae), *Catenotaenia henttoneni* (Cestoda: Catenotaeniidae), *Mastophorus muris* (Nematoda: Spirocercidae), *Hymenolepis horrida* (Cestoda: Hymenolepididae), *Trichuris arvicolae* (Nematoda: Trichuridae) and *Syphacia petrusewiczi* (Nematoda: Oxyuridae). Details concerning the prevalence levels are presented in Table 1.

Table 1
Parasitological data concerning the 98 bank voles sampled

Parasites	Code	*N*	Prevalence (CI)
Viruses			
Puumala virus	PUUV	9	9.2 (4.87–15.48)
Cowpox virus	CPXV	15	15.3 (9.67–22.59)
Macroparasites and coccidia			
Neotrombicula inopinata	Mites	48	49.0 (40.27–50.74)
Frenkelia glareoli	Cocc-Fg	1	1.0 (0.05–4.75)
Echinococcus multilocularis	Cest-Em	11	11.2 (6.42–17.90)
Taenia taeniaeformis	Cest-Tt	29	29.6 (22.04–38.09)
Paranoplocephala gracilis	Cest-Pg	9	9.2 (4.87–15.48)
Catenotaenia henttoneni	Cest-Ch	16	16.3 (10.51–23.74)
Mastophorus muris	Nem-Mm	13	13.3 (8.02–20.27)
Hymenolepis horrida	Nem-Hh	5	5.1 (2.03–10.43)
Trichuris arvicolae	Nem-Ta	3	3.1 (0.83–7.73)
Syphacia petrusewiczi	Nem-Sn	4	4.1 (1.40–9.10)

Cocc, Cest and Nem, respectively, refer to coccidia, cestodes and nematodes. *N* is the number of seropositive (viruses) or infected (macroparasites, protozoans) animals. Prevalence estimates are presented in percentage. CI corresponds to the 95% confidence interval of prevalence estimates.

3.2. DQA genotyping

The screening of DQA polymorphism using CE-SSCP for the 98 bank voles sampled revealed nine different SSCP patterns. Seventeen individuals were cloned and 136 clones were sequenced to constitute the DNA bank. After applying the criteria described by Kennedy et al. (2002), we defined nine SSCP patterns (alleles) corresponding to nine different sequences of the complete *DQA* exon 2. Nomenclature is Clgl-*DQA* as already used in Bryja et al. (2006) and proposed by Klein et al. (1990). The sequences of the *DQA* exon 2 are available in GenBank under the following accession nos. EU008329, EU008330, EU008331 and EU008332. Phylogenetic analyses resulted in a single shortest tree for both nucleotides (not shown) and amino acids (Fig. 1). All exon 2 sequences identified translated in unique amino acid sequences. This reflects the high level of non-synonymous substitutions at this gene. As previously described in Bryja et al. (2006), one to four different alleles could be observed for a single bank vole,

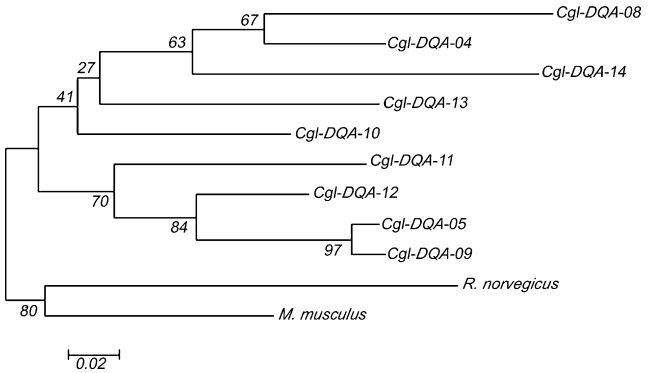

Fig. 1. Neighbour-joining phylogenies for the nine *M. glareolus* amino acid sequences using *Rattus norvegicus* and *Mus musculus* sequences as outgroups. The scale bar represents the genetic distance and the numbers are bootstrap values (5000 replicates).

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Table 2
Clgl-*DQA* allele's frequencies in bank voles trapped in Mignovillard

Clgl-*DQA* alleles	Frequencies (%)
4	3
5	16
8	83
9	11
10	16
11	21
12	7
13	1
14	14

$N = 98$. Frequencies are calculated in terms of number of individuals presenting alleles compared to the total dataset.

indicating both the duplication of the *DQA* gene in *M. glareolus* and the intra-population polymorphism of this duplication. Based on the phylogenetic analyses, it was not possible to assign the nine different alleles to a particular copy of the *DQA* gene. The duplication event in *M. glareolus* MHC probably took place after the separation of Cricetidae and Muridae (see also Bryja et al., 2006). Therefore, the duplication polymorphism was not included as a measure of *DQA* diversity in the following analyses. Allelic frequencies, estimated from the presence/absence of each allele per individual, are detailed in Table 2.

3.3. Statistical analyses

The coccidium *Frenkelia glareoli* and the allele Clgl-*DQA*13 were removed from the association analysis because they were only found in one bank vole.

The first two dimensions of the COA explained 39% of the variance of genetic data (first factor F1: 21%; second factor F2: 18%). The projection of the genetic variables on the F1/F2 plane revealed that heterozygosis, Cgl-*DQA*-08 and Cgl-*DQA*-10 were located close to the origin, and thus did not discriminate bank voles (Fig. 2A). The alleles Cgl-*DQA*-05 and Cgl-*DQA*-11 or Cgl-*DQA*-04 were located in opposite directions on F1 and exhibited the highest factorial values. They have therefore antagonist effects. The alleles Cgl-*DQA*-05 and Cgl-*DQA*-14 were opposed on F2 (Fig. 2A). Fig. 2B shows the PCA correlation circle for the parasitological variables projected on the F1/F2 plane. These first two axes accounted for 32% of the variance, with 17% explained on the first factor F1 and 15% on the second one F2. The first factor opposed bank voles infected with different helminths while the second factor (F2) divided voles in a group including PUUV seropositive ones and another group including voles infected with mites. Note that most of the variables were located near the centre of the factor map, which means that they have low factorial values.

These independent results were coupled using ACO. The global co-inertia between the genetic and parasitological matrices reached 32%. After 10,000 permutations, 2547 co-inertia estimates were larger than the one observed in our data. This indicates a poor global relationship between the two matrices. We further analysed particular associations between PUUV and *DQA* gene polymorphism. The two first axes of the

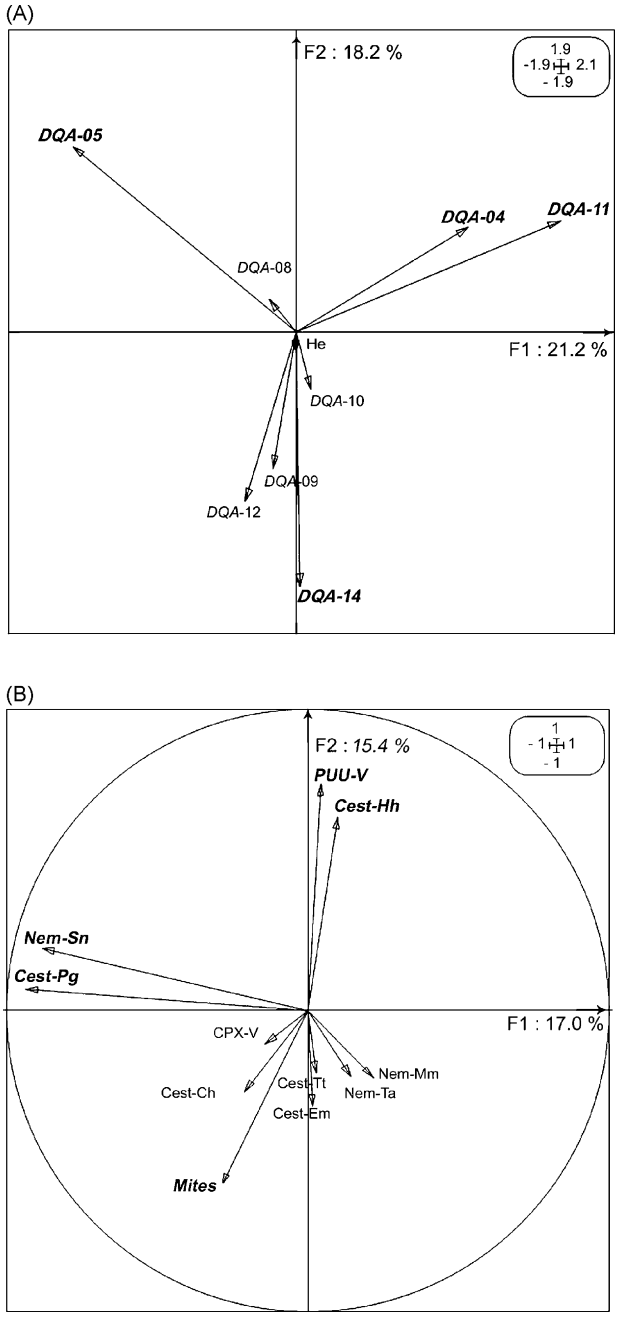

Fig. 2. Multivariate analyses of the genetic and parasitological matrices analysed independently. Variables are projected on the factor map defined by factor 1 (F1) and factor 2 (F2). F1 explains most of the variance and F2, which is orthogonal to F1, explains the most of the remaining variance. For each analysis, the variance explained by each factor is indicated in percentage. For each pair of axes, the variables located in a given direction relative to the origin can be considered positively associated, whereas the variables located in opposite directions can be considered antagonistic. The main variables explaining the total variance are edited in bold. Variables plotted near the origin cannot be interpreted. (A) Correspondence analysis (COA) of *DQA* gene polymorphism in the 98 bank voles. The *DQA* alleles, coded Cgl-*DQA*-XX, and heterozygosity (Het), are projected on the F1/F2 map. (B) Correlation circle (F1 × F2) of the principal component analysis (PCA) performed on the parasitological matrix. Nem and Cest, respectively, indicate nematode or cestode. The two following letters refer to the code used to identify the species (see Table 1).

co-inertia represented 58% of the variance (31% F1 and 27% F2). Fig. 3A shows the ACO factor map for the genetic variables. The distribution of genetic variables on this map was almost similar to the one observed on the previous COA factor map (Fig. 2A). Two changes could be noticed. First, although the Cgl-*DQA*-10 allele has not been important in the COA, it strongly structured the second factor of the ACO. Second, the Cgl-*DQA*-12 and Cgl-*DQA*-05 have been negatively associated on the second factor of the COA, but they were positively associated on the first factor of the ACO. Fig. 3B shows the distribution of the parasitological variables on the F1 × F2 ACO factor map. PUUV infection played a role in the co-inertia between genetic and parasitological matrices as PUUV serological status structured the F2 axis, with CPXV serological status. A surprising result was the opposition between PUUV infection and the presence of mites. This opposition was previously observed from the PCA analysis. Major changes between the PCA and ACO factor maps can be noted. CPXV antibodies appeared important in the co-inertia analysis although they did not structure the PCA factor map. Inversely the cestode *H. horrida*, which was important in the PCA and positively associated with PUUV infection, was no more important in the co-inertia analysis.

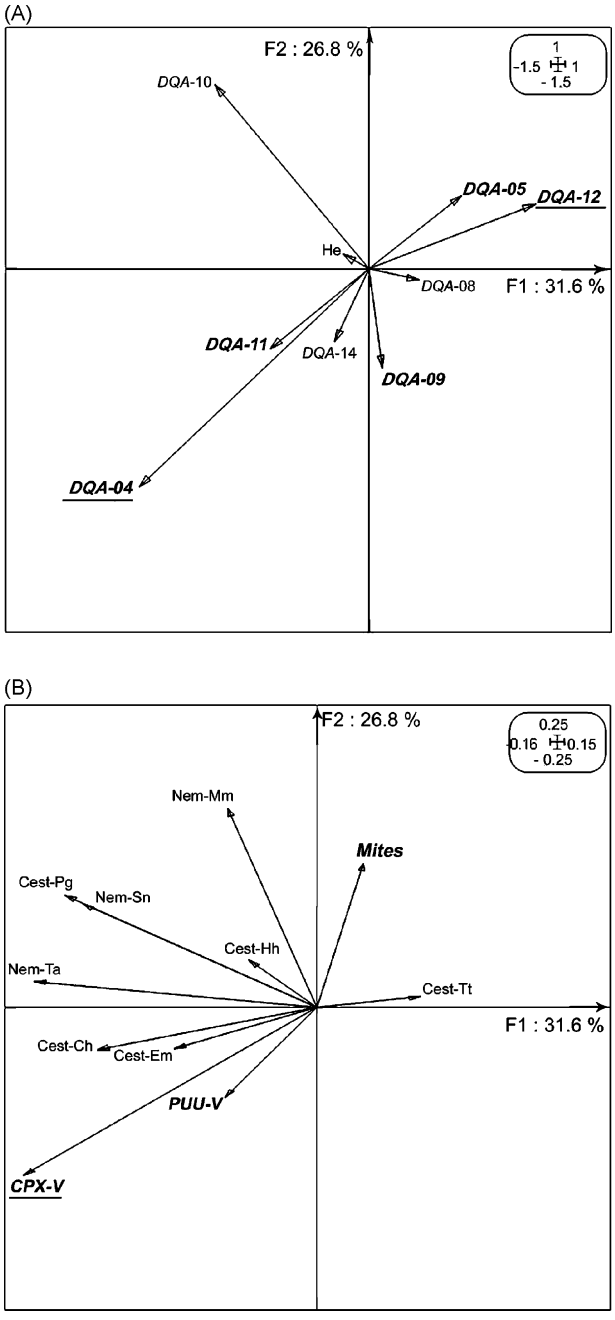

Fig. 3. Distribution of (A) the genetic variables encoded as in Fig. 2A, and (B) the parasitological variables encoded as in Fig. 2B on the first factor map (F1 × F2, defined as in Fig. 2) of the ACO. Under strong co-inertia, the variables located in a given direction relative to the origin can be considered positively associated, whereas the variables located in opposite directions can be considered antagonistic. The genetic and parasitologic variables associated with PUUV in the ACO are edited in bold style. Those associated with CPXV are also underlined.

The comparison between Figs. 3A and B revealed that the main genetic variables associated with PUUV antibodies are Cgl-*DQA*-05 and Cgl-*DQA*-12 (negative associations, RR = 0.21 and 0.57, respectively: individuals carrying these alleles have respectively a 0.21 and 0.57 times lower risk to get infected), and in a lesser extent Cgl-*DQA*-09 and Cgl-*DQA*-11 (positive association, RR = 2.83 and 2.07). This comparison also stressed the strong genetic–parasitological associations between CPXV antibodies and both Cgl-*DQA*-04 (positive association, RR = 10.18) and Cgl-*DQA*-12 (negative association, RR = 0.32). This supports the important role of CPXV serological status in the co-inertia between parasitism and MHC polymorphism. Details concerning the number of bank voles within each of these categories are provided in Table 3.

Interestingly, all four alleles mainly implicated in the association with PUUV antibodies, Cgl-*DQA*-05, Cgl-*DQA*-12 and Cgl-*DQA*-09 clustered in a well-sustained clade (bootstrap value >85, Fig. 1). Despite their phylogenetic proximity, the three alleles differed by ten amino acid sites (over a total of 81 sites). Moreover, 5 amino acid substitutions occurred within the 11 sites, which were previously found to be under positive selection in rodents (see Bryja et al., 2006). The two closest alleles, Cgl-*DQA*-05 and Cgl-*DQA*-09, which were involved in antagonistic associations with regard to PUUV, were separated by only two amino acid changes, one of them belonging to a site under positive selection.

4. Discussion

In humans, the clinical severity of NE depends on the MHC genetic background of the patients, with haplotypes associated to a benign clinical course and other with a severe one (Mustonen et al., 1996, 1998, 2004; Plyusnin et al., 1997). Investigating the existence of such associations in reservoirs of hantaviruses is thus particularly important to better understand the risks of HFRS emergence related to wild rodents populations.

4.1. Associations between PUUV and DQA alleles

Our study revealed associations between a class II MHC gene and PUUV infection in bank voles. More precisely, we

Table 3
Correspondence table between genetic factors selected from the co-inertia analysis and (A) Puumala virus antibodies or (B) Cowpox virus antibodies

	Cgl-*DQA*-09		Cgl-*DQA*-05		Cgl-*DQA*-12		Cgl-*DQA*-11	
	Presence	Absence	Presence	Absence	Presence	Absence	Presence	Absence
(A) Puumala virus antibodies								
Presence (%)	2 (18.19)	7 (8.04)	0 (0)	9 (10.98)	0 (0)	9 (9.89)	3 (14.28)	6 (7.79)
Absence (%)	9 (81.81)	80 (91.96)	16 (100)	73 (89.02)	7 (100)	82 (90.11)	18 (85.72)	71 (92.21)
RR	2.83		0.21		0.57		2.07	

	Cgl-*DQA*-04		Cgl-*DQA*-12	
	Presence	Absence	Presence	Absence
(B) Cowpox virus antibodies				
Presence (%)	2 (66.67)	13 (13.68)	0 (0)	15 (16.48)
Absence (%)	1 (33.33)	82 (86.32)	7 (100)	76 (83.51)
RR	10.18		0.32	

The number in each cell shows the sample size. Seroprevalence distribution (percentage of seronegative and seropositive individuals) is given both for individuals with and without each genetic factor identified. RR corresponds to the relative risk associated with each allele, estimated from Haldane (1956). Alleles are grouped in the table depending on the sign of the association (positive: Cgl-*DQA*-09 and 04 or negative: Cgl-*DQA*-05 and 12); Cgl-*DQA*-11 is also positively associated with PUUV antibodies but in a lesser extent (see ACO, Fig. 3).

detected a positive association between PUUV antibodies and the allele Cgl-*DQA*-09, and negative associations with the alleles Cgl-*DQA*-05 and Cgl-*DQA*-12. Considering the allele Cgl-*DQA*-09, two individuals exhibit both the alleles and PUUV antibodies. There was a high proportion of seronegative voles that did not carry this allele, as well as a low proportion of voles carrying this allele and being seronegative. This suggests that voles carrying the Cgl-*DQA*-09 allele could be more susceptible than those which did not carry it. Alternatively, none of the PUUV seropositive voles carried either the alleles Cgl-*DQA*-05 (nine individuals) or Cgl-*DQA*-12 (nine individuals). This could be interpreted as a ''resistance'' role of these alleles in PUUV infection. However, this interpretation requires confirmation through experimental infection as PUUV seronegative individuals might be resistant voles or voles which have not yet met the virus.

In humans, the class I HLA-B8, the class II *DRB**0301 and *DQ2* alleles are associated with a severe course of NE (Mustonen et al., 1996). The precise mechanisms underlying these associations are yet not well described but different hypotheses have been developed (review in Terajima et al., 2004). Individuals that carried this extended haplotype have impaired lymphocyte activation and an imbalance in cytokine production (Mustonen et al., 1998). It has been proposed that the antiviral response either may be dysfunctional and fail to clear the virus, or may also function with inappropriate efficacy: the HLA-B8-DRB3 haplotype is associated with autoimmune diseases that may have a viral etiology. Molecular mimicry could explain the association of this haplotype with the renal disease caused by Puumala virus infection (Plyusnin et al., 1997).

However, these associations might be indirect, if this polymorphism is in linkage disequilibrium with a functional polymorphism in other genes located within the MHC or that predispose voles to PUUV infection. A potential candidate is the TNF-α gene, located within the class III region of the MHC. Increased levels of TNF-α are known to induce pathophysiological and clinical changes similar to those seen in NE. Polymorphism in the promoter region of this gene is thus likely to be associated with the severity of NE. A recent study showed that patients carrying the TNF-2 allele, which is associated with enhanced TNF-α transcriptional activity, suffered from a more severe NE (Mäkelä et al., 2002). However, it is likely that this result is due to strong linkage disequilibrium with the HLA haplotype previously mentioned. The HLA polymorphism seems to be a more important risk factor for severe NE than the TNF-α polymorphism (Mäkelä et al., 2002).

Experimental infections and immunological surveys need to be investigated in the bank vole to confirm the existence of resistant/susceptible individuals or the variability in the outcome of the infection with PUUV, and to study the relative influence of DQA polymorphism versus other gene polymorphism on PUUV infection.

4.2. No associations between PUUV and DQA heterozygosis

The alternative hypothesis potentially underlying associations between MHC polymorphism and PUUV infection is overdominance. Doherty and Zinkernagel (1975) suggested that heterozygous individuals at MHC loci could have an immunologic advantage because of their ability to present a wider range of foreign peptides to T cells. Under this scenario, we expected heterozygous voles to be less parasitized than homozygous ones. Our results do not provide evidence in favour of this heterozygote advantage hypothesis. Nevertheless, we have to be cautious with these results. Our dataset concerns a single site and might not be large enough to cover the whole pathogen community. The absence of associations between heterozygosity and parasitism might thus be confirmed before concluding about the importance of overdominance as a mechanism of selection acting on DQA gene in bank voles.

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4.3. Antagonist infections with mites and PUUV

Analysing PUUV infections jointly with the whole helminth community, viruses and protozoan infections gave us the opportunity to detect antagonist and/or synergistic effects of parasites on the PUUV/MHC associations observed. In this study, the multivariate approach developed on parasitological variables revealed a strong opposition between mites and PUUV infections. Among the nine PUUV seropositive voles, only two were infected by mites. Alternatively, among the PUUV seronegative voles, the proportions of individuals infected and non-infected by mites were similar (respectively, 51% and 48%). This contrast between mites and PUUV infections was still observed when analysing the co-structure between parasitological and genetic variables. This suggests an antagonist role of immunogenetics in resistance/susceptibility to mites and PUUV. To our knowledge, this is the first time that this antagonism is highlighted. It is quite surprising as a role of trombiculid mites in hantavirus transmission has even been suggested (Song, 1999; Houck et al., 2001). Several explanations might be envisaged and tested experimentally: (i) one of the immune response developed against PUUV infection and associated with MHC susceptibility factor in voles might prevent from further mite infestations, (ii) one of the immune pathway associated with MHC factor of resistance to PUUV infection might not prevent from further mite infestations, and (iii) trombiculid mites are vectors of microparasites, which were not detected in this study. The immune response developed against these microparasites might prevent further infestations with PUUV. This hypothesis is worth investigating as trombiculid mites are known vectors of bacteria such as *Borrelia* sp. (Kampen et al., 2004) or *Rickettsia* sp. (Frances et al., 2000).

4.4. Associations between Cowpox virus and DQA polymorphism

Surprisingly, our results show that CPXV plays an important role in the co-structure between parasitological and MHC data in bank voles. The two main associations detected in this study concerned the allele Cgl-*DQA*-04, which is rare but found in over 60% of CPXV-seropositive individuals, and the allele Cgl-*DQA*-12, which was never found among the 15 CPXV-seropositive individuals. This might suggest that this allele is associated with resistance to CPXV.

Orthopoxviruses, along with a number of other viruses, share mechanisms capable of modulating the host immune response. Many orthopoxviruses possess a protein that is capable of down-regulating MHC class I, which is responsible for presenting all intracellular antigens (Dasgupta et al., 2007). Biochemical and functional analyses have also revealed that poxviruses infection directly interfered with ligand binding to class II molecules (Ping et al., 2005). Therefore, down-regulation of MHC class I trafficking and disruption of MHC class II-mediated antigen presentation may be some of the multiple strategies orthopoxviruses have evolved to escape host immune surveillance. This modulation of the host defence mechanisms seems to rely on homologues of immune molecules encoded by orthopoxviruses (Johnston and McFadden, 2003). Combining immunogenetics of genes involved in host immunity and virus evasion could help us understanding these associations.

5. Conclusions

Our study provides evidence for the role of immunogenetics in viral infections. Several alleles are suggested for either susceptibility or resistance to these infections. However, these results have to be taken cautiously as they concern few seropositive individuals. Moreover, as demonstrated for passerines and malaria parasites, links between host immunogenetics and resistance can result from local adaptation processes (Bonneaud et al., 2006). The associations detected might thus involve population-specific alleles and consequently, vary in space and/or time. Further investigations are needed to confirm the associations observed and to test the existence of such spatio-temporal fluctuations.

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