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Short communication

Experimental infections of wild bank voles (*Myodes glareolus*) from nephropatia epidemica endemic and non-endemic regions revealed slight differences in Puumala virological course and immunological responses

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ABSTRACT

In Europe, the occurrence of nephropathia epidemica (NE), a human disease caused by Puumala virus (PUUV), exhibits considerable geographical heterogeneity despite the continuous distribution of its reservoir, the bank vole *Myodes glareolus*. To better understand the causes of this heterogeneity, wild voles sampled in two adjacent NE endemic and non-endemic regions of France were infected experimentally with PUUV. The responses of bank voles to PUUV infection, based on the levels of anti-PUUV IgG and viral RNA, were compared. Slight regional differences were highlighted despite the high inter-individual variability. Voles from the NE non-endemic region showed greater immune responsiveness to PUUV infection, but lower levels of RNA in their organs than voles from the endemic region. These results suggest the existence of regional variations in the sensitivity of bank voles that could contribute to the apparent absence of PUUV circulation among voles and the absence of NE in the non-endemic region.

Puumala virus (PUUV) is an enveloped tri-segmented RNA virus in the *Hantavirus* genus (family *Bunyaviridae*), of which the natural reservoir is the bank vole *Myodes glareolus*. In humans, PUUV is responsible for nephropathia epidemica (NE), a zoonotic disease provoked by inhalation of virus-containing aerosols from contaminated rodent excreta (Lundkvist and Niklasson, 1992). Although the bank vole is found in most areas of France except on the Mediterranean coast, human cases are only being reported in the northeast (Olsson et al., 2010). In addition to this heterogeneous geographic distribution of PUUV, experimental infections and field surveys have demonstrated some variability in the probability of a bank vole being infected with PUUV (Deter et al., 2008; Kallio et al., 2006; Olsson et al., 2002) and in the pattern of PUUV excretion once infected (Hardestam et al., 2008; Voutilainen et al., 2015). More investigations are required to better understand this heterogeneity and its consequences on PUUV distribution among bank vole populations. An artificial comparison of the outcomes of *M. glareolus*/PUUV interaction in bank voles from endemic (human cases of NE) and non-endemic regions (absence of NE cases) could be particularly useful. Bank voles from non-endemic regions might be less competent for PUUV infection and thus strongly limit virus circulation within their populations as well as the risk of NE human cases. By contrast, bank voles from endemic regions might be more competent for PUUV infection. This could result in higher levels of PUUV replication and excretion in the environment, and therefore lead to increased risks of bank vole infections and human cases of NE.

This hypothesis can be tested by experimentally infecting bank voles sampled in endemic and non-endemic regions, and examining their immune responses and PUUV kinetics through time. Up to now, such studies have only been performed on colonized voles, bred in the laboratory for several generations (Hardestam et al., 2008; Kallio et al., 2006; Yanagihara et al., 1985). However, unlike the situation in wild animals, factors such as inbreeding, housing conditions or diet in laboratory-bred voles can lead to changes in intrinsic characteristics (immune responsiveness and resistance to diseases, gut microbiome and

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its capacity to synthesize vitamins impacting immune functions), that are likely to affect the outcomes of *M. glareolus*/PUUV interactions (Kau et al., 2012; Keller and Waller, 2002).

In this study, bank voles were captured in two neighboring French regions, with contrasting statuses in terms of human cases of NE, and were experimentally infected. The number of human cases of NE has been increasing in Jura since 2005, making this region an endemic focus (National Reference Center for Hantavirus; Mailles et al., 2005). No human case has ever been reported in Ain, a non-endemic region. Genetic differences between the two bank vole populations should be limited as they both belong to the western European lineage, are geographically close (about 100 km between the two sampling sites) and there is no apparent spatial barrier separating the two regions. Bank voles were sampled from an area of a few km^2 in each region during fall 2015, using seed-baited live-traps (Supplementary Table 1). After three weeks in quarantine (see Yanagihara et al., 1985; Hardestam et al., 2008), 22 seronegative voles were transferred to an ABSL-3 facility and kept in individual ISOcages N (Tecniplast). Food and water were provided *ad libitum*. Based on the results of preliminary experiments, we decided to use 1.7×10^3 focus forming units (f.f.u) of the prototype PUUV (Sotkamo strain) diluted in DMEM (ThermoFisher Scientific) for injection by subcutaneous route into 10 bank voles from each region. The Finnish Sotkamo strain has been cell culture adapted after numerous passages on Vero E6 Strain. However, it has already been used in several *in vivo* experimental infections (Lokugamage et al., 2003; Sanada et al., 2011). PBS or heat-inactivated virus (60 °C, 60 min) were injected subcutaneously into two bank voles per region to serve as negative controls. Blood, saliva, urine and feces were collected from each bank vole at three days post-infection (dpi). Approximately 200 µL of whole blood were sampled through the retro-orbital sinus and stored at 4 °C before centrifuging for sera collection 24 h later. Saliva was collected on sterile swabs subsequently placed in 300 µL of Hank's Balanced Salt Solution (Life Technologies) and vortexed for 10 s. Urine samples and feces were also collected. All samples were stored at −80 °C until analysis. Two infected bank voles from each region were euthanized each week, and the controls were euthanized at the end of the experiment (35 dpi). Blood, excreta, bladder, spleen, kidney, liver, lungs and salivary glands were collected and stored at −80 °C. Blood and excreta were also collected once a week from the controls and from half of the remaining infected bank voles, to limit the impact of manipulations on vole response.

Serum samples were screened by IgG ELISA as described in Klingstrom et al. (2002). Because of the high cross-reactivity between different hantavirus serotypes (Krüger et al., 2001), the plates were coated with Tula virus-infected and non-infected cell lysate. These antigens have been validated in previous experiments with a panel of negative and positive bank vole sera. Anti-PUUV positive and negative sera reacted similarly with lysates of PUUV and TULV infected cells as well as recombinant PUUV nucleocapsid protein (data not shown). The presence of neutralizing antibodies was detected by performing a focus reduction neutralization test (FRNT) using Sotkamo strain, as described in Niklasson et al. (1991). Viral RNA was extracted from all samples using the QIAamp Viral Mini Kit (Qiagen). Quantitative RT-PCR was performed with 2.5 µL of viral RNA amplified by applying the SuperScript III One-Step RT-PCR system with Platinum Taq High Fidelity (Invitrogen) on a LightCycler 480. All samples were tested in duplicate to avoid false positives and negatives. Relative amounts of viral RNA (expressed in RNA copy per mg of tissue) were calculated using a standard curve obtained with *in vitro* transcribed RNA. Nested RT-PCR, which is more sensitive than quantitative RT-PCR, was applied using the Titan One Tube RT-PCR system (Roche) and Taq DNA Polymerase (Qiagen). Primers, probe sequences and cycling conditions are detailed in Supplementary Table 2.

None of the four control bank voles seroconverted and no viral RNA was detected in their excreta or organs. In the infected bank voles, seroconversion occurred between 14 and 28 dpi (Table 1). Possible variation in the amount of IgG produced between regions and over time was tested with a linear mixed model, using the lme function implemented in the nlme package for R 3.1.0 (R Core Team, 2013). The 10 bank voles that had seroconverted at least by 14 dpi were included in the model. The dependent variable was the optical density measured at 450 nm ($OD_{450\ nm}$). The fixed variables included region, dpi and their interaction. Bank vole identity was included as a random effect. Chi-square tests with Bonferroni correction were applied to analyze the effect of significant variables using the package *phia* for R. The model revealed a higher $OD_{450\ nm}$ in bank voles from the PUUV non-endemic region than in bank voles from the endemic region ($X_1^2 = 4.124$, $p = 0.042$). The *dpi* * *region* interaction was also significant ($X_6^2 = 17.68$, $p = 0.007$), with inter-regional differences in $OD_{450\ nm}$ being higher at 28 dpi ($X_1^2 = 9.1910$, $p = 0.017$). Neutralizing antibodies were detected between 14 and 35 dpi in bank voles from both regions. Optical densities and titers were slightly higher in bank voles from Ain (50 to 200) than in those from Jura (50 to 100).

Viral RNA was detected by nested RT-PCR in bank vole sera from Jura between 3 and 7 dpi but only at 7 dpi in those from Ain. Using qRT-PCR, viral RNA could be detected in voles from both regions and in any organs among those tested, i.e. lungs, liver kidney and salivary glands (Table 2). A mixed linear model was applied as previously described to see if viral RNA content in the lung, which is the target organ for PUUV (Bernshtein et al., 1999; Gavrilovskaya et al., 1983) varied between regions and time. Viral RNA loads were significantly higher in bank voles from Jura, the PUUV endemic region ($t = 3.167$, $p = 0.007$). The *dpi* * *region* interaction was also significant ($t = -3.114$, $p = 0.008$), with higher levels of viral RNA in lungs of bank voles from the PUUV endemic region than in those from the PUUV non-endemic region at until 14 dpi and the opposite pattern at 28 dpi. Joint analysis of the anti-PUUV IgG in serum and the viral RNA in organs revealed a diverse array of patterns, indicating that the presence of antibodies was not always correlated with the presence of viral RNA in the organs (Tables 1 and 2). Finally, no viral RNA was detected in the excreta, even when these were associated with infected organs.

One originality of this project was the experimental infection of wild rodents as other investigations of hantaviruses have involved laboratory or colonized animals (e.g. (Hardestam et al., 2008; Kariwa et al., 1996; Yanagihara et al., 1985). This made it possible to avoid the effects of inbreeding that might artificially affect the outcome of PUUV infection. One of the most important results in these experimental infections is that inter-individual variability in the virological course and immunological responses to PUUV infection was high in both populations, as previously described in natural populations (Bernshtein et al., 1999; Glass et al., 1998; Mills et al., 1997). Diverse patterns were demonstrated regarding the different organs in which viral RNA was detected in the same bank vole, the time to seroconversion and the combined presence of antibodies and viral RNA between bank voles. Viral RNA was present in most organs of some individuals, while in others only one or two organs were infected. One individual did not seroconvert or exhibit any viral RNA, but whether this was due to an experimental error or the result of a real biological process could not be determined. Five voles did not seroconvert although PUUV RNA was detected in some of their organs. This delayed production of antibodies has already been observed in natural populations. For example, (Voutilainen et al., 2015), using a capture-mark-recapture longitudinal survey of bank voles, showed that PUUV RNA could be detected in the excreta seven weeks before seroconversion occurred. This strong inter-individual variability can be mediated by many factors in addition to the classical characteristics of sex (Bernshtein et al., 1999; Hannah et al., 2008), age (Arikawa et al., 1986; Mills et al., 1999) and immunogenetics (see Charbonnel et al., 2014 for a review). Such factors could not be examined in this study due to the small number of voles that can be handled simultaneously in animal facilities. In particular, the wild bank voles in our study may have differed in their prior exposure to parasites or access to resources. These important