Prolonged survival of Puumala hantavirus outside the host: evidence for indirect transmission via the environment

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The capability of rodent-borne viruses to survive outside the host is critical for the transmission dynamics within rodent populations and to humans. The transmission of Puumala virus (PUUV) in colonized bank voles (*Clethrionomys glareolus*) was investigated and additional longevity studies in cell culture with PUUV and Tula (TULV) hantaviruses were performed. Wild-type PUUV excreted by experimentally infected donor bank voles was shown to be transmitted indirectly between rodents through contaminated beddings, and maintained its infectivity to recipient voles at room temperature for 12–15 days. In cell culture supernatants, PUUV and TULV remained infectious for 5–11 days at room temperature and up to 18 days at 4 °C, but were inactivated after 24 h at 37 °C. Interestingly, a fraction of dried virus was still infectious after 1 h at 56 °C. These results demonstrated that hantavirus transmission does not require direct contact between rodents, or between rodents and humans, and that the indirect transmission of PUUV through contaminated environment takes place among the rodents for a prolonged period of time. The results also have implications for safety recommendations for work with hantaviruses and for preventive measures.

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INTRODUCTION

The survival of pathogens outside the host is important for the epidemiological dynamics of several disease agents. The persistence in the environment is highly important for the circulation of *Hepatitis A virus* (Crance *et al.*, 1998), *Foot-and-mouth disease virus* (Thomson *et al.*, 2003), parvovirus (Barker & Parrish, 2001), as well as for infectious canine hepatitis virus (Woods, 2001) and avian influenza viruses (Stallknecht *et al.*, 1990). However, only limited data are available concerning the potential of rodent-borne viruses, transmitted without an intermediate vector, to remain infectious outside their natural hosts.

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Many rodent populations undergo strong density fluctuations. The temporal low host-population density is critical for long-term persistence of rodent-borne viruses. The capability of viruses to avoid extinction depends on the host-population dynamics and on the specific characteristics of the host–virus interaction. Recently, Sauvage *et al.* (2003) highlighted the importance of potential survival of Puumala hantavirus (PUUV) outside the rodent host for its persistence and transmission dynamics. Mathematical modelling suggested that an indirect mode of transmission via the natural environment and prolonged survival outside the host was required to generate an epidemic pattern compatible with the one observed (Sauvage *et al.*, 2003). Prolonged contagiousness outside the host may facilitate the persistence of viruses during a temporal loss of the infection in the host population. Such persistence in the

environment has also been speculated to play a role in the dynamics of *Cowpox virus*, another rodent-borne virus (Begon *et al.*, 2003). Thus, the longevity in the environment might be a common adaptive characteristic of rodent-borne viruses, transmitted without intermediate vectors, in maintaining endemic infection dynamics.

Hantaviruses (family *Bunyaviridae*) are enveloped, three-segmented, negative-stranded RNA viruses. Hantaviruses have co-evolved with their rodent hosts through millions of years and each hantavirus has its own specific rodent host species (for a review, see Vapalahti *et al.*, 2003). PUUV and its host, the bank vole (Brummer-Korvenkontio *et al.*, 1980), are found in most of Europe. *Tula virus* (TULV) is carried by the European common vole (*Microtus arvalis*) (Plyusnin *et al.*, 1994) and is distributed widely in central and eastern Europe. PUUV is the causative agent of *nephropathia epidemica*, a mild form of haemorrhagic fever with renal syndrome, with thousands of diagnosed cases in Europe annually, although TULV is believed to be non-pathogenic for humans (Vapalahti *et al.*, 2003).

Hantavirus infections have not been reported to cause any apparent clinical illness in the carrier rodents (Bernshtein *et al.*, 1999; Botten *et al.*, 2002; Compton *et al.*, 2004; Gavrilovskaya *et al.*, 1990; Hutchinson *et al.*, 1998; Lee *et al.*, 1981). Hantavirus infection is chronic (for review, see Meyer & Schmaljohn, 2000) and the transmission is horizontal. Infectious PUUV is secreted in urine, faeces and saliva and the shedding starts approximately 1 week after infection (Gavrilovskaya *et al.*, 1990). Although hantaviruses are transmitted via indirect and direct contacts (Bernshtein *et al.*, 1999; Botten *et al.*, 2002; Gavrilovskaya *et al.*, 1990; Kariwa *et al.*, 1998; Lee *et al.*, 1981; Nuzum *et al.*, 1988; Yanagihara *et al.*, 1985), the relative importance of the different modes of transmission is not known. The resistance of hantaviruses against environmental conditions has been studied mainly in the context of inactivation procedures (Johnson, 1989; Schmaljohn *et al.*, 1998). However, more information on the longevity of hantaviruses is needed for a better understanding of the epidemiological patterns, both in rodent and human populations.

Here, we have studied the survival of PUUV outside its host by exposing bank voles to beddings of infected donor bank voles (*in vivo* experiments). As the first results showed an unexpectedly long time-period of indirect transmission of PUUV between bank voles, we extended the *in vivo* experiments, and tested the survival of hantaviruses in cell culture. In these *in vitro* experiments, PUUV and TULV were exposed to different temperatures, either in cell culture medium or after drying.

METHODS

***In vivo* experiments.** A total of 156 colonized bank voles was used in the experiments. The laboratory colony was established from PUUV-free animals captured in Sweden in the 1990s (Lundkvist *et al.*, 1996).

Throughout the experiment, no direct contact was allowed between individuals. The voles were held individually in polycarbonate rearing cages ($28 \times 17 \times 12$ cm) with wood shavings as bedding. The cages were in HEPA-filtered isolators, at room temperature (approx. 22 °C) and humidity between 40 and 60 %. The distance between cages was more than 15 cm, which is enough to prevent the intercage transmission of PUUV in the isolators (our unpublished observations). The photoperiod was constant: 16 h light and 8 h dark. Commercial mouse pellets and water were provided *ad libitum*. Fresh apples and lettuce were supplied once a week. For rearing conditions and handling of the animals, the guidelines and animal experimentation permits of the Swedish Institute for Infectious Disease Control (SMI) were followed.

The experiment was run in two trials. In the first trial, there were 15 donor individuals and five recipient groups (RGr) (15 recipients per group, altogether 75 recipient individuals). In the second trial, there were six donors and 60 recipient individuals divided into 10 RGr to prolong the study. The recipient individuals were divided randomly into RGr, taking, however, into account the gender and the age of available individuals. On day 0, the donor bank voles were inoculated subcutaneously with 100 bank vole ID_{50} of PUUV strain Kazan-wt-II (passaged twice in colonized bank voles). They were held individually in cages in isolators. On day 10, each donor was moved into a new separate clean experimental cage. On day 17, the donors were removed from the experimental cages and they were bled, sacrificed and sampled for tissues. Subsequently, also on day 17, the first RGr (RGr1) were moved individually into the experimental cages and exposed to the donors' beddings for 3 days (until day 20), when they were moved into clean management cages (individually) for 15 days to develop the infection. The second RGr (RGr2) individuals were put into the experimental cages for the next 3 days (from days 20 to 23). The procedure was continued in the same manner for all RGrs (in trial 1 until RGr5 and in trial 2 until RGr10). After 15 days in management cages, the animals were bled, sacrificed and dissected, tissues were sampled, weights measured and sexual maturation status checked (for females: open or closed vagina and uterus size; for males: testicle size).

Each of the experimental cages was occupied every day during the study, but no direct contact was allowed between any individual during the experiment, e.g. the fifth RGr individuals were in the experimental cages from days 12 to 15 and the individuals of RGr10 from days 27 to 30 following removal of the donor animals.

The 3 day period of exposure for the recipients was chosen based on the results found in the literature: shedding or transmission of hantaviruses in less than 5 days post-infection has not been reported. The earliest transmission of PUUV to other bank voles was reported by Gavrilovskaya *et al.* (1990) to occur 5 days after infection. The observed start of transmission and/or shedding of other hantaviruses depended on the study: e.g. Yanagihara *et al.* (1985) reported PUUV transmission 2 weeks after infection, Lee and his co-workers (1981) detected Hantaan virus in urine 7 days post-inoculation, Botten *et al.* (2002) found Sin Nombre virus RNA from saliva samples starting at day 15 post-infection, Kariwa *et al.* (1998) detected Seoul virus RNA on day 7 post-infection and Hutchinson and her collaborators (1998) reported *Black Creek Canal virus* in saliva 7 days post-infection. To confirm that the recipient individuals did not shed the virus while they were in the experimental cages, urine samples from the RGr1 individuals were collected when they were removed from the experimental cages. All these samples were negative for viral RNA by RT-PCR (see below). Therefore, the 3 day stay in the experimental cages was assumed to be short enough to prevent the shedding of the virus in any way by the recipient individuals.

Fourteen female and seven male bank voles were used as donors. The age of the donors at inoculation varied between 8 and 21 weeks (mean 15 weeks). The recipients were 7–24 weeks old (mean 15 weeks) when they were exposed to the secreted virus. Among the recipients, 57·5 % were females, 42·5 % males and 92 % were sexually mature.

Detection of PUUV infection. PUUV infection was studied using a nested RT-PCR to detect PUUV S-segment RNA in lung and urine samples from bank voles, as described earlier (Plyusnin *et al.*, 1997). RNA extraction from bank vole lung tissue and urine was carried out using the Ultraspec RNA isolation system (Biotech Laboratory) according to the manufacturer's instructions. Serum samples were screened for IgG antibodies using an IgG enzyme-immunoassay (EIA) as described earlier (Klingström *et al.*, 2004).

Statistical analyses. We used Pearson χ^2 test, Fisher's exact test and Student's *t*-test in data analyses in SPSS for Windows.

***In vitro* experiments.** In the *in vitro* experiment, the longevity of PUUV and TULV was studied in cell culture supernatants at different temperatures. The incubations were done either in the cell culture medium (designated below as 'wet') or after drying on glass surfaces of microscope spot slides (designated below as 'dry') (Fig. 1). PUUV strain Sotkamo (Vapalahti *et al.*, 1992) and TULV strain Moravia (Vapalahti *et al.*, 1996), both adapted to Vero E6 cell culture, were used.

The viruses were diluted in minimum essential medium with 10 % fetal calf serum, L-glutamine and antibiotics, and prepared as a 10-fold series from undiluted to a 1/10 000 dilution. In the dry experiment (Fig. 1), 10 µl of each virus dilution was applied on spots of two sterilized 10-spot-slides in duplicate. The virus suspension was subsequently air-dried for 15 min in a biosafety laminar hood at room temperature. In the wet experiment (Fig. 1), 100 µl of each dilution was put into 1·5 ml microtubes (one microtube per temperature per time period). Both the slides of dry-treated samples and the microtubes of wet-treated samples were stored in closed 50 ml tubes during the incubation. The samples were incubated at temperatures of −20, 4, 23, 37 and 56 °C for various time periods. After the incubation, 10 µl of each wet sample was applied to two sterilized slides (two wells per slide) as described above. Immediately afterwards, 30 µl cell suspension (containing approximately 48 000 Vero E6 cells) was applied to the wet samples. The same amount of cells was applied on the dried samples (two slides per temperature per time period) after the incubation. The slides were held in a humid incubation chamber at 37 °C (5 % CO_2) during the infection. After 46 ± 6 h, the slides were washed with PBS, air-dried and fixed with ice-cold acetone. Infected cells were detected by an immunofluorescence assay as described earlier (Vapalahti *et al.*, 1995).

Slides were screened by fluorescence microscopy using a 400-fold magnification. Altogether 20 microscopic fields (in two wells on two slides) were observed randomly for each of the studied dilutions. Based on the initial titrations, optimal virus dilutions were selected for further studies; the PUUV samples were undiluted [producing $6{\cdot}9 \times 10^5$ focus-forming units (f.f.u.) ml^{-1}], the TULV samples from the incubation temperatures −20, 4, 23 and 37 °C were either undiluted or diluted 1/10 (producing $8{\cdot}8 \times 10^5$ f.f.u. ml^{-1}), and undiluted for the samples that were incubated at 56 °C. The proportion of cell coverage in each of the observed fields of vision was approximated and the number of virus-positive foci in the microscopic fields was counted. Only fields with more than 50 % of confluence were counted. For each field with < 100 % cell coverage, the number of positive foci was recalculated to represent 100 % cell coverage. The results shown in Figs 2 and 3 represent a mean of 20 vision fields.

RESULTS

In vivo experiments

The virus was transmitted to recipients through the beddings contaminated by the donors. Infected voles were found in the RGr1–5, which were exposed for up to 15 days after the donors were removed (Table 1). As no end point for the survival of PUUV outside the host was found in the first trial (isolators 1–3), a second, extended trial was performed (isolators 4–6). Altogether, there were 10 RGr in the second trial and none of the voles in RGr6–10 (days 15 to 30) was infected. PUUV remained infectious outside the host for up to 12–15 days. There was no difference in the proportion of infected individuals in the different RGr (Table 1).

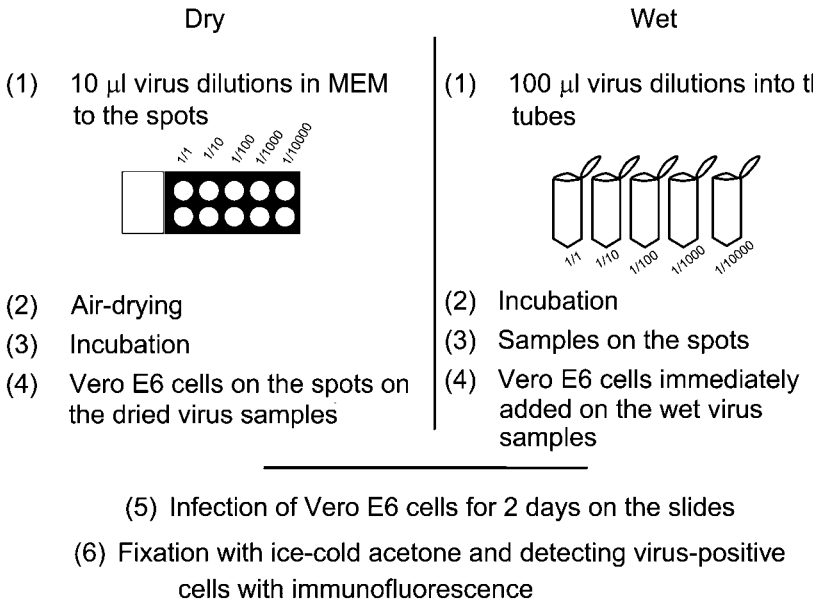

Fig. 1. Outline of the cell culture experiments.

Table 1. Bank voles used in the *in vivo* experiments

Infected individuals (detected by RT-PCR) are in bold. RGrs are shown with the number of days since removal of the donors in parentheses. During these periods the RGr individuals were exposed to the donors' beddings. F, female; M, male. The numbers after F or M represent the age of the animals (in weeks), at which they were exposed to the donors' beddings.

Isolator	Donor individual	RGr1 (0–3)	RGr2 (3–6)	RGr3 (6–9)	RGr4 (9–12)	RGr5 (12–15)	RGr6 (15–18)	RGr7 (18–21)	RGr8 (21–24)	RGr9 (24–27)	RGr10 (27–30)	No. infected/ total
1	1: F 18	F 21	**F 12**	**F 21**	**F 22**	F 22	–	–	–	–	–	3/5
	2: F 18	**F 21**	**F 18**	**M 18**	**M 21**	**F 21**	–	–	–	–	–	5/5
	3: F 15	F 18	**F 21**	F 21	**M 19**	**F 19**	–	–	–	–	–	3/5
	4: F 12	F 15	**M 23**	F 13	**M 19**	**F 19**	–	–	–	–	–	3/5
	5: F 11	**F 12**	F 21	**F 12**	**M 15**	F 15	–	–	–	–	–	3/5
2	6: F 11	**F 12**	M 12	**F 18**	F 21	M 21	–	–	–	–	–	2/5
	7: M 21	M 24	F 20	M 22	F 22	F 22	–	–	–	–	–	0/5
	8: M 17	M 20	F 12	M 20	M 15	F 15	–	–	–	–	–	0/5
	9: M 15	M 18	M 18	**M 12**	F 15	F 15	–	–	–	–	–	1/5
	10: M 15	M 12	M 20	F 12	F 16	F 16	–	–	–	–	–	0/5
3	11: F 12	**F 10**	F 10	F 11	**F 10**	F 7	–	–	–	–	–	2/5
	12: F 11	M 7	F 7	M 10	F 7	**F 11**	–	–	–	–	–	1/5
	13: F 11	M 9	**F 11**	**M 8**	F 11	**M 10**	–	–	–	–	–	3/5
	14: F 9	F 10	M 8	F 7	M 8	M 8	–	–	–	–	–	0/5
	15: F 8	F 8	M 9	F 9	M 9	**M 9**	–	–	–	–	–	1/5
4	16: F 17	F 21	F 19	F 14	**M 20**	F 15	F 13	M 18	M 13	M 14	F 10	1/10
	17: F 17	M 11	M 14	F 21	F 22	**F 20**	M 12	M 9	F 11	M 12	M 13	1/10
5	18: M 17	**F 16**	**M 19**	F 19	F 11	M 12	F 10	M 10	M 12	F 18	M 14	2/10
	19: M 17	M 19	*	M 11	M 11	F 11	F *	M 12	F 14	M 13	F 17	0/10
6	20: F 17	F 11	M 18	**M 18**	F 20	M 11	F 18	F 13	F 12	F 11	F 10	1/10
	21: M 17	M 16	F 21	M 19	M 19	M 19	M 16	F 19	M 20	F 11	M 11	0/10
	Total 21/21	5/21	6/21	7/21	7/21	7/21	0/6	0/6	0/6	0/6	0/6	32/135

*Data of individual characteristics (sex and/or age) are lacking.

All 21 donors were PUUV-RNA positive 17 days after virus inoculation as determined by RT-PCR of lungs (Table 1), and 90 % had seroconverted. Of the 135 recipients, 32 were positive as determined with RT-PCR (Table 1), and nine of them had seroconverted 17 days after the first contact with the donor beddings. All urine samples from the RGr1 animals were negative by RT-PCR at the end of the exposure (day 3). Donors 12, 15 and 17 infected individuals only from the fifth RGr, which were exposed to the donors' beddings from days 12 to 15, showing that the recipients were exposed to the virus shed by the donors, and not by virus shed by infected individuals in the previous RGr in the experimental cages.

Possible risk factors for infection of the recipients were further studied in the RGr1–5. Of these 105 individuals, 59 % were females and 41 % were males. Neither sex, nor maturation status of the recipients had a significant effect on the infection risk (gender: $\chi^2 = 0{\cdot}28$, d.f. = 1, $P = 0{\cdot}60$, maturity: Fisher's exact test $P = 0{\cdot}77$). There was no difference in the weight or age between the infected and uninfected recipients (mean weight $\pm$ SD, infected: $22{\cdot}1 \pm 3{\cdot}5$ g ($n = 31$), uninfected: $21{\cdot}9 \pm 4{\cdot}3$ g ($n = 69$), t = 0·16, d.f. = 98, $P = 0{\cdot}87$; mean age $\pm$ SD in weeks, infected: $16{\cdot}2 \pm 4{\cdot}6$ ($n = 32$), uninfected: $14{\cdot}9 \pm 5$ ($n = 72$), t = 1·19, d.f. = 102, $P = 0{\cdot}28$). The significance of the results did not differ when the recipients ($n = 35$), which were exposed to donors that did not transmit the virus further (donors 7, 8, 10, 14, 19 and 21), were omitted from the analyses.

Altogether, 15 of 21 donors transmitted the virus to a recipient. Female donors infected the recipients more often than males (Fisher's exact test $P = 0{\cdot}006$). Of 14 female donors, 13 transmitted the virus further, whereas only two of seven male donors transmitted PUUV to recipients.

In vitro experiments

Cell-culture grown PUUV and TULV were exposed to different temperatures, either in cell culture medium or after drying, and the amount of infectious virus was quantified at different time points. The titre of the original input virus was $6{\cdot}9 \times 10^5$ or $8{\cdot}8 \times 10^5$ f.f.u. ml^{-1}, producing approximately 35 or 45 foci per visual field for PUUV (undiluted) or TULV (1/10 dilutions), respectively (Fig. 2a and b). When the viruses were dried at room temperature before incubation (the same titres as in 'wet' treatment), they lost most of their infectivity (Fig. 2c and d).

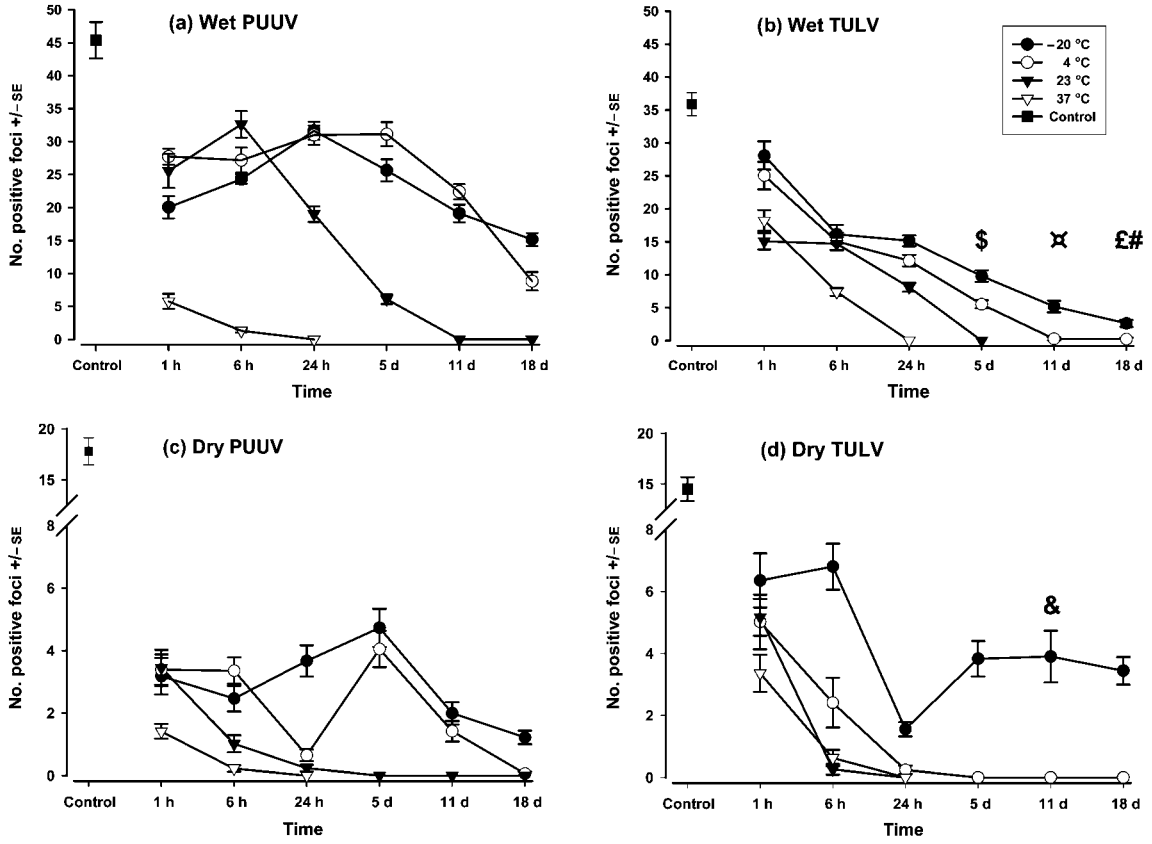

Fig. 2. Infectivity of PUUV and TULV after incubation at different temperatures. Lines represent the mean number of foci of PUUV (undiluted samples) and TULV (1/10 diluted samples). The viruses were either in cell culture medium (a and b) or dried (c and d) during the incubation at the indicated temperatures. Symbols ($, ¤, £, # and &) represent the treatments and the time points at which the undiluted TULV foci were counted (mean ± SE): $ (represents incubation temperature 23 °C) = 1·38 ± 0·41; ¤ (4 °C) = 1·06 ± 0·30; £ (4 °C) = 1·15 ± 0·43; # (−20 °C) = 27·07 ± 2·67; & (4 °C) = 0·05 ± 0·01.

As expected, PUUV and TULV were stable when frozen at −20 °C. Furthermore, both viruses were still infectious after 18 days of incubation in cell culture medium at 4 °C (Fig. 2). At room temperature (23 °C) the viruses lost their capability to infect cells in 24 h when dried, but were still infectious after 5 days in a wet environment. At 37 °C, the viruses were completely inactivated within 24 h. Fifteen minutes at 56 °C was enough to inactivate viruses in cell culture medium. However, when the viruses were dried before incubation at 56 °C, a fraction of them remained infectious for 1 h (Fig. 3). Dried TULV was completely inactivated within 2 h at 56 °C.

DISCUSSION

To our knowledge, this is the first time longevity of naturally shed hantavirus outside the host has been studied. Our results demonstrated that PUUV remained infectious in bank vole cage beddings for 12–15 days at room temperature after removal of the infected animals. Although transmissions resulting from indirect contacts between rodents

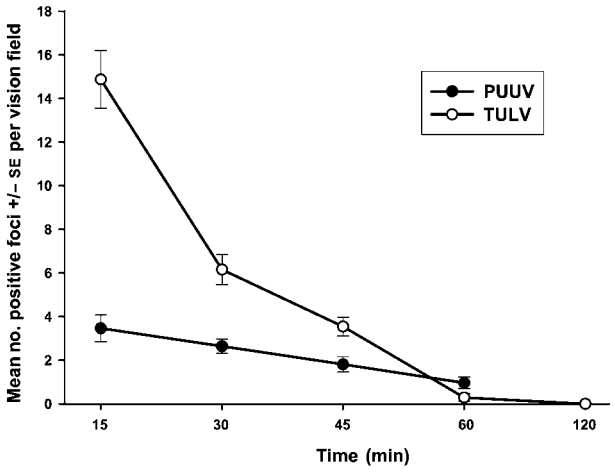

Fig. 3. Infectivity of PUUV and TULV (foci counted from undiluted samples) after incubation at 56 °C. The viruses were dried before incubation.

have been demonstrated earlier for some hantaviruses, e.g. PUUV (Gavrilovskaya *et al.*, 1990), Seoul (Kariwa *et al.*, 1998) and Hantaan (Lee *et al.*, 1981), we demonstrated that the indirect transmission occurs over an extended time-period. The results highlight the importance of PUUV survival outside the rodent host for transmission dynamics and human risk. Mathematical modelling by Sauvage *et al.* (2003) suggested that an indirect mode of transmission via a contaminated environment and prolonged virus survival outside the host was required to generate an epidemic pattern compatible with the observed one for PUUV (Sauvage *et al.*, 2003). Interestingly, the authors used an estimation of 1 week for the survival of PUUV (Sauvage *et al.*, 2003), which correlates well with our experimental results. The recipients in the present study were exposed to relatively freshly inoculated donors that had received a high inoculum, which may have resulted in an increased virus production during the early stages of infection. In a natural situation, persistently infected voles with a lowered virus production might be more numerously represented than those recently infected.

The role of indirect (aerosols) and direct (physical contacts) transmission modes in hantavirus dynamics, as well as the main route of the shedding of hantaviruses, have been discussed extensively and the results have been rather conflicting (e.g. Botten *et al.*, 2002; Gavrilovskaya *et al.*, 1990; Hinson *et al.*, 2004; Hutchinson *et al.*, 1998; Kariwa *et al.*, 1998; Klein *et al.*, 2000, 2001, 2004; Meyer & Schmaljohn, 2000; Padula *et al.*, 2004; Vapalahti *et al.*, 1999). For an efficient transmission via saliva, direct contacts are needed, since the amount of saliva secreted into the environment is likely to be very low. Transmission via saliva is often thought to be connected with aggressive behaviour and biting between rodents. In contrast, shedding of the virus in urine and faeces would enhance the probability of indirect transmission, since the amounts of secreted urine and faeces exceed that of saliva. Most likely both direct and indirect transmissions are involved in the infection dynamics of hantaviruses, but the importance of the different modes could vary depending on the hantavirus species, host-population dynamics, seasonal demographical features and related changes in behaviour of the host species, and on the environment. Different functional groups in rodent populations (breeding females and males, non-breeding subadults, juveniles) show very different levels of aggression and activity.

In addition, transmission strategies among the various hantaviruses may differ (Hutchinson *et al.*, 1998). Different kinds of optimal persistence and transmission strategies may have evolved during the long co-evolution between hantaviruses and their rodent hosts (Vapalahti *et al.*, 1999). The distribution ranges of PUUV and TULV cover mostly boreal and temperate regions where environmental conditions could be selective for prolonged survival.

We could not find any differences in the infection rates of recipient voles due to age, weight, sex or the maturation status. In field studies, characteristics like age, weight and breeding status of bank voles have been found to influence the PUUV seroprevalence (Bernshtein *et al.*, 1999; Escutenaire *et al.*, 2000, 2002; Niklasson *et al.*, 1995; Olsson *et al.*, 2002; Verhagen *et al.*, 1986, 1987). Although the reports on gender-related differences have not been systematical, higher infection rates in old-breeding males have been observed in some studies (Bernshtein *et al.*, 1999; Escutenaire *et al.*, 2002; Olsson *et al.*, 2002). On the other hand, we found that female donors infected more recipients than male donors. It remains to be seen if this is true in nature, in spite of the higher seroprevalence in breeding males.

Indirect transmission makes the transmission rate less dependent on the host density and enhances the persistence of virus in the host population (Sauvage *et al.*, 2003); the northern Europe bank vole populations vary cyclically with deep decline phases (crashes), which last for more than a year (Hanski *et al.*, 1994; Hansson & Henttonen, 1988). During crashes, the bank vole density may be extremely low, clearly less than one vole per hectare (Prévot-Julliard *et al.*, 1999). The prolonged survival of PUUV is likely to enhance its transmission, especially during these low bank vole densities. Moreover, PUUV might persist in an area despite the temporary loss of infectious bank voles from the population (Sauvage *et al.*, 2003) or even a temporary loss of the host individuals themselves.

Our results showed that the longevity of PUUV and TULV depends on the temperature and moisture. In nature, there are additional physical and chemical factors, like UV light, sunlight and pH, which may have an influence on the longevity of viruses. However, during the winter in northern Europe, voles spend most of their time under the snow cover in rather stable environmental conditions. A thick snow cover maintains a stable temperature and humidity, and protects secreted viruses from the UV light. Thus, we assume that the significance of the prolonged longevity of PUUV is more important during winter than during summer, and in northern Europe than in more southern and drier zones. In addition to the geographical differences in rodent dynamics and habitat fragmentation, environmental factors influencing the survival of PUUV may partly explain the regional differences in the human epidemiology of *nephropathia epidemica* in Europe. In northern Europe there are thousands of diagnosed cases annually, whereas in temperate, more populated Europe, the annual number of human cases is only some hundreds (Vapalahti *et al.*, 2003).

The infectivity of viruses in cell culture medium was lost within 5–11 days at 23 °C, which is in line with the 12–15 days survival of PUUV observed in the animal experiments. The slightly longer survival of PUUV shed by bank voles compared with virus in cell culture might be attributable to a different pH or protein content, or due to a higher virus titre; the titre of PUUV excreted by our experimental animals is unknown. Additionally, since the infectivity of PUUV in bank vole decreases after passage in Vero

E6 cell culture (Lundkvist *et al.*, 1997), it is possible that other properties also change during cell culture adaptation.

The inactivation time of PUUV and TULV in cell culture depended on whether they were dried or not. Both viruses were inactivated within a 15 min incubation at 56 °C in cell culture medium that is compatible with the present recommendations for hantavirus heat inactivation, 30 min at 60 °C (Johnson, 1989). Based on the results presented in Schmaljohn *et al.* (1998), dried Hantaan virus remained viable for several days at room temperature. Our results indicated that drying the viruses decreased the infectivity considerably (Fig. 2), but a fraction still remained infectious and may be more resistant to heat. Thus, the increased stability of dried viruses should be taken into consideration when these viruses are inactivated.

The prolonged survival in the environment is a well known phenomenon in the epidemiology of some viral pathogens. However, this is the first time prolonged survival has been proven for hantaviruses. Prolonged survival of rodent-borne viruses outside the host is likely to increase the fitness of the virus and may therefore be selected for at natural dynamical conditions. Rodents represent the only evolutionary setting for hantaviruses: no arthropod vector is known – unlike for other members of the family *Bunyaviridae* – and human infections have no role in hantavirus epizootiology. Conceivably, the prolonged survival could well have been a significant fitness factor developed and selected for during the co-evolution of hantaviruses for millions of years together with their rodent hosts. It will be of interest to learn whether other rodent-borne viruses, such as arenaviruses, share this property and whether hantaviruses differ from the other arthropod-borne genera of the family *Bunyaviridae*.

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