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Short communication

Puumala and Tula hantaviruses in France

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Abstract

The first genome sequences of Tula (TULV) and Puumala (PUUV) hantaviruses undoubtedly originated from France were recovered from tissue samples of European common voles and bank voles captured in Jura region. Genetic analysis of S and M segments of French PUUV strain revealed its highest similarity to strains from neighboring Belgium and Germany and also from Slovakia. On phylogenetic trees, French PUUV strain was placed within the central European lineage formed by strains from these three countries. Both of our French TULV strains clustered together and formed a distinct, well-supported genetic lineage.

Keywords: RNA viruses; Hantaviruses; Puumala virus; Tula virus; Phylogenetic analysis

Hantaviruses (genus *Hantavirus*, family *Bunyaviridae*) are negative-strand RNA viruses with a tripartite genome, each carried by a specific rodent host. Some hantaviruses are human pathogens causing Haemorrhagic Fever with Renal Syndrome (HFRS) and Hantavirus (Cardio)Pulmonary Syndrome, while others are apathogenic (Nichol et al., 2005). Three hantaviruses, namely Puumala (PUUV), Dobrava (DOBV) and Saaremaa (SAAV), are known to cause HFRS in Europe (Vapalahti et al., 2003). PUUV, a major European human pathogen, causes a generally milder than HFRS disease, called *Nephropathia epidemica* (NE), which presents most commonly with fever, headache, gastrointestinal symptoms, impaired renal function and blurred vision. Since 1980, over 1000 cases of hantavirus-caused human infection have been registered in France (Mailles et al., 2005). The HFRS/NE endemic areas are in the north-eastern France: in the Ardennes, along the Belgian border and also along the German border (Mailles et al., 2005; Sauvage et al., 2002). Hantavirus infections have been reported also in Ile-de-France (the Paris area) (Lautrette et al., 2003) and Jura (near the Swiss border) (Mailles et al., 2005). The natural host of PUUV, bank vole *Clethrionomys glareolus*, is widely distributed on the territory of France, except some areas on the Mediterranean cost (Mitchell-Jones et al., 1999). So far, no PUUV genome sequences have been recovered from bank voles trapped in France and only one, very short, partial S segment sequence has been recovered from a Belgian NE-patient who was presumably infected in French Pyrenean mountains (region of Perpignan) (Keyaerts et al., 2004).

Three more rodent species known to carry hantaviruses exist in France: (i) *Apodemus flavicollis*, the host of DOBV, (ii) *Rattus norvegicus*, the natural host of Seoul virus (SEOV), and *Microtus arvalis*, a carrier of TULV (Vapalahti et al., 2003). The first two hantaviruses are established human pathogens, TULV considered non-pathogenic. First SEOV genome sequences originated from France (Lyon) were recently reported (Heyman et al., 2004). There are no reports on DOBV or TULV in France as yet.

Recently (2005), several rodent species from the French Jura were screened for hantaviral markers [Deter et al., Ms in preparation]. Eight bank voles *C. glareolus* and nine common voles *Microtus arvalis* were found positive for hantavirus-specific antibodies. These were further screened by immunoblotting for the presence of hantaviral nucleocapsid (N) protein antigen, and that has been detected in four *C. glareolus* and two *M. arvalis*. Then these six samples were analyzed using RT-nested PCR. Two *C. glareolus* and two *M. arvalis* were found positive. The aim of this study is to gain more insights into the relationships between French PUUV strains and other PUUV (and PUUV-

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like) strains from Eurasia and also between French TULV strains and strains from other European countries. Toward this aim we recovered the complete S segment and partial M segment sequences of PUUV from *C. glareolus* and also partial S segment sequences of TULV from *M. arvalis* captured in Jura region and subjected them to phylogenetic analysis.

RNA was extracted from rodent lung tissue samples using the TriPure RNA isolation system (Behringer Maannheim). Partial S-sequences (nt 819–1082) of PUUV were obtained by RT-nested PCRs (Plyusnin et al., 1997). PCR-amplicons were gel-purified using QIAquick Gel Extraction kit (QIAGEN) and sequenced automatically using ABI PRISM^{TM} Dye Terminator sequencing kit (Perkin-Elmer/ABI, NJ). The partial S-sequences were 100% identical and, indeed, belonged to PUUV. Corresponding wild-type PUUV strain (which has not been isolated) was designated as PUU/Mignovillard/CgY02/2005 or MignovillardY02, for short. Next, using a different RT-PCR strategy (Plyusnin et al., 1994) we recovered complete S segment sequence from the tissue sample of one of these bank voles; partial M segment sequence (nt 2161–2570) was recovered from this sample as well. All experimental details are available upon request. Both sequences showed the highest similarity to known PUUV strains from Belgium, Germany and Slovakia.

To infer phylogenies, the PHYLIP program package (Felsenstein, 1993) was used. Hantavirus sequences used for comparison were recovered from the GenBank. Five hundred bootstrap replicates generated for complete coding sequences of the S segment, as well as partial sequences of the S segment and the M segments (Seqboot program) were fed to the distance matrice algorithm (Dnadist). Distance matrices were analyzed with the Fitch-Margoliash tree-fitting algorithm (Fitch) or with NJ-algorithm (Neighbor); the bootstrap support values were calculated with the Consense program. The nucleotide sequence data were also analyzed using maximum likelihood approach (with the Tree-Puzzle program) (Schmidt et al., 2002). On phylogenetic trees constructed for the complete coding region of the S segment (nt 43–1344) French PUUV strain was indeed placed into the central European (CE) lineage formed by strains from Belgium, Germany and Slovakia. Fitch-Margoliash tree is shown in Fig. 1A; the Neighbor-joining and Puzzle trees exhibited essentially the same branching pattern. Such a placing was well supported (bootstrap support value of 99%). Within the CE-lineage, the French PUUV strain was located outside the well-supported group that included strains from Belgium (Momignies47 Momignies55, Couvin59, Thuin33, Montbliart23, Belgium13891, 14444 and 14445) and Germany (CgErft).

Altogether, nine genetic lineages of PUUV can be seen on the S-tree. In addition to eight previously established (Plyusnina et al., 2006) genetic lineages: Danish (DAN), CE, Alpe-Adrian (ALAD), Russian (RUS, which includes also strains from Estonia), Finnish (FIN, which includes also strains from Russian Karelia and Siberia (Omsk region)), South Scandinavian (S-SCA) and North Scandinavian (N-SCA that consist of strains from Sweden and Norway), and Japanese (JPN that includes *C. rufocanus*-associated strains from Hoikkaido), there is now the recently recognized Korean lineage (KOR) represented by *Eothenomys regulus*-originated Muju strains (Song et al., 1999). It is quite possible that JPN and KOR lineages represent, in fact, distinct hantavirus species, Hokkaido virus (HOKV) and Muju virus (MUJV), respectively. The lineages are well supported, with the sole exception of the S-SCA lineage (bootstrap support value of 68%; however, this value was substantially higher, 95%, on the neighbor-joining tree). The overall phylogeny is star-like: relationships between the lineages remain obscure.

To include in our analysis the previously reported short PUUV sequence recovered from a NE-patient who was presumably infected in southern France (Keyaerts et al., 2004), the phylogenetic tree was calculated for the corresponding part of the S segment (nt 43–293). The fragment of this tree is shown in Fig. 1B. Two French PUUV strains, the bank vole-originated strain MignovillardY02 and the NE-patient-originated strain Perpignan, were placed within the CE genetic lineage of PUUV but, surprisingly, not in the closest proximity to each other. While the Perpignan strain was located within the cluster formed by Belgian and German strains, the MignovillardY02 strain occupied the outsider's position. There might be two possible explanations for this result: (i) PUUV strains from the French Pyrenean Mountains and from Jura have different evolutionary histories; (ii) the Belgian NE-patient was infected back in his home country. It should be noted that the phylogeny based on rather short S-sequences is less reliable than the phylogeny inferred for the complete coding S-sequences. For instance, the bootstrap support value for the CE-lineage is far below the confidence level of 70% (Hillis and Bull, 1993). Therefore, the results of this analysis should be treated with caution.

On the phylogenetic tree constructed for partial M segment sequences (Fig. 2), the French PUUV strain was, again, placed into the CE lineage (bootstrap support value of 77%) and, within the lineage, it was located outside the well-supported duo of strains from Belgium and Germany. Seven genetic lineages of *bona fide* PUUV can be easily recognized of the M-tree: DAN, ALAD, RUS, FIN, S-SCA and N-SCA (the M segment sequences for Japanese and Korean strains have not been determined yet). Interestingly, the M-phylogeny is not purely star-like. The CE and ALAD lineages share a common ancestor and so do the RUS and FIN lineages. This observation suggests somewhat closer evolutionary relationships between CE and ALAD lineages and also between RUS and FIN lineages.

Partial S segment sequences (nt 918–1215, primers' sequences excluded) recovered from two *M. arvalis* belonged to TULV and were 97.3% identical. Deduced amino acid sequences of the encoded part of the N protein were 100% identical. Corresponding wild-type TULV strains (which have not been isolated) were designated as TUL/Mignovillard/MaY27/2005 and TUL/Mignovillard/Ma1751/2005 or MignovillardY27 and Mignovillard1751, for short. Our attempts to recover complete S segment sequences from TULV-positive rodent tissue samples were not successful. On phylogenetic trees two French TULV strains clustered together and formed a distinct, well-supported genetic lineage (The Fitch-Margoliash tree is shown in Fig. 3; Neighbor-joining and Puzzle trees revealed same branching pattern). Four other distinct, well-supported lineages of TULV can