Title

First isolation and genetic characterization of Puumala orthohantavirus strains from France

Authors

Johann Vulin[γ*1], Séverine Murri*[1], Sarah Madrières[1,2], Maxime Galan[2], Caroline Tatard[2], Sylvain Piry[2], Gabriele Vaccari[3], D'agostino Claudia[3], Nathalie Charbonnel[2], Guillaume Castel[2], Philippe Marianneau[1]

Affiliations

[1]Laboratoire de Lyon, ANSES, Unité de virologie, Lyon, France

[2]CBGP, INRAE, CIRAD, IRD, Institut Agro, Univ Montpellier, Montpellier, France

[3]Department of Food Safety, Nutrition and Veterinary Public Health, Istituto Superiore di Sanità, 00161 Rome, Italy

* Those authors contributed equally to the work.

[γ] Corresponding mail : johann.vulin@anses.fr

Keywords

Hantavirus, Puumala, isolation

Abstract

Puumala orthohantavirus (PUUV) causes a mild form of hemorrhagic fever with renal syndrome (HFRS) named nephropathia epidemica (NE), regularly diagnosed in Europe. France represents the Western frontier of NE expansion in Europe with two distinct areas: the endemic area (Northeast) where PUUV circulates in rodent populations and where many cases of NE are detected in humans and non-endemic area (Southwest) where the virus is not detected and only a few human cases have been reported. The country is a pertinent target to study factors that influence the evolution of PUUV distribution. In this study, we describe for the first time the isolation of two PUUV strains from two distinct French geographical areas: Ardennes (endemic area) and Loiret (non-endemic area). To isolate PUUV efficiently, we selected wild rodents (*Myodes glareolus*, the specific reservoir of PUUV) from these areas that were seronegative for anti-PUUV IgG (ELISA) but associated with viral RNA load in lung (qRT-PCR). With this design, we are able to cultivate and maintain these two strains in VeroE6 cells but also to propagate efficiently and rapidly both strains in a bank vole colony. Complete coding sequences of S and M segments were determined by Sanger sequencing of RNA extracted from

positive bank voles (naturally and experimentally infected) and from supernatant of Vero E6. For the M segment, nucleotidic sequences were 100% identical for both strains. For the S segment, the amino acid sequences from each strain revealed one mismatch between sequences obtained from tissue and from supernatant, revealing a “bank vole” and a “cell” signature. High throughput sequencing confirmed Sanger results, and provided a better assessment of the impact of isolation methods on intra-host viral diversity.

Text

Orthohantaviruses represent an increasing threat to humans due to their worldwilde distribution, the increase of infection number and the emergence or re-emergence of new viruses (1). Puumala virus (PUUV) is the main orthohantavirus circulating in France. This tri-segmented enveloped RNA virus, hosted by the bank vole (*Myodes glaerolus*), is responsible for a mild form of hemorrhagic fever with renal syndrome (HFRS) named nephropathia epidemica (NE) (2). About 100 cases of NE are reported annually in the Northeast part of France (3).

In France, sequences of PUUV have been studied so far from bank voles samples (4) and recently from human samples (5) but isolated strains have never been cultured or maintained until now. Considering the adaptation capacity of PUUV to cell culture and changes in infectivity induced by cell culture passages (6), having a “wild-type” virus is a crucial step in the thorough study of the biology of this virus. Unfortunately, because of its slow growth in cell culture, the isolation of PUUV is often very difficult.

In this study, we tried to determine a method for selecting bank voles containing live virus in order to isolate PUUV strains. Seto et al (7) were able to isolate PUUV from bank voles that harbored orthohantavirus nucleocapsid protein (NP) in their lung and had no antibody against PUUV. This combination probably corresponds to the active viremic phase. We have adapted this approach by selecting, in our collections of bank vole samples captured in different French forests during the 10 last years (4) (8), seronegative animals with high amounts of viral RNA

in lungs. Serum samples were screened using IgG ELISA as already described (8) and viral RNA was extracted from lung homogenates using QIAamp Viral Mini Kit according to the manufacturer's instructions (Qiagen). Quantitative RT-PCR was performed with 2.5 µL of viral RNA amplified by applying the Super- Script III One-Step RT-PCR system with Platinum Taq High Fidelity (Invitrogen) on a LightCycler 480 (Roche).

More specifically, we carried out these methods on bank voles trapped in two distinct French geographical areas. A NE endemic area: the Ardennes, where PUUV circulates in rodent populations and where many cases of NE are detected in humans (3). Then a non-endemic zone: the Loiret where PUUV circulates in rodent populations and where no or very few cases of NE are diagnosed (4). We were able to select seven bank voles trapped in Ardennes during the autumn 2011 (among a total of 201 trapped animals in this session) and one bank vole trapped in Loiret in the summer 2014 (among 44 animals). For our first isolation assays, we used the sample named Vouzon/2014 (Loiret) that exhibited a CP of 23.65 cycles in lung and an animal from Ardennes named Hargnies/2011 with the smallest CP: 18.90 cycles.

From these two animals, we tried to isolate live viruses from lung homogenates (5% weight/volume). We combined many conditions such as centrifugation (with or without), filtration (0.22µM) and dilution (10^{-1} to 10^{-4}) for homogenate preparation, cell confluence and numbers of cell passages before infection (16 or 43) and different infection methods between the three passages before isolation (supernatant, transposition or co-culture). The conditions that led to the *in vitro* isolation of the two viral strains consisted in the use of the lung homogenate centrifuged (300 rpm 10 minutes), not filtrated, diluted at 10^{-1} and applied on low-passaged Vero E6 cells (16 passages) in DMEM Dulbecco′s Modified Eagle′s – Medium) 5% SVF (Gibco) and cultured at hyper-confluence ($1x10^6$ cells for one cupule of P6). It was necessary to proceed to three successive passages to reach a sufficient quantity of live viruses.

These passages were realized using “co-culture”: a quarter of the initially infected cells were transferred in a culture of non-infected cells ($2x10^5$ cell / cupule P6) accompanied by 500 µL of the supernatant from the infected culture added to 2 mL of fresh media.

The viral load measured in supernatant of veroE6 cells increased notably: -15.2 CP and -12.8 CP for Ardennes - Hargnies and Loiret - Vouzon respectively **(Figure 1A)**. Moreover, at the end of cell isolation, viral titer was $1.6x10^4$ PFU/mL and $1.5x10^4$ PFU/mL for Ardennes - Hargnies and Loiret - Vouzon respectively. It should be noted that their focus size when titrated were different from PUUV control the Sotkamo strain serially passaged in VeroE6 cell **(Figure 1B)**. The focus size of cultivated viruses was smaller (in particular for Loiret - Vouzon) than Sotkamo focuses. Phenotypical variations have already been described for PUUV titration assay (9). Altogether, these results showed that we were able to select viral isolates from natural host tissue and to cultivate them in cell culture.

Furthermore, we carried out an *in vivo* isolation process that consists of biological assays on bank voles that were maintained at ISS institute. We used the same lung homogenates from trapped animals already used for *in vitro* isolation process. We performed a subcutaneous injection of lung homogenate (5% weight/volume) on two animals for each strain. None of the inoculated animals showed clinical evidence of viral infection. Seven days after infection, we collected blood samples through retro-orbital sinus puncture and carried out PUUV serological detection and viral RNA analyses. We did not detect any anti-PUUV antibody (**Table 1**) but we found viral RNA in all sera analyzed. Viral loads were similar whatever the PUUV strain considered (Ardennes - Hargnies versus Loiret - Vouzon). This indicated that our protocol lead to an effective transmission of PUUV in these animals. Two days later, at nine days post infection, we euthanized the four bank voles by cervical dislocation. We found that all animals had seroconverted with a higher antibody titer observed for Loiret - Vouzon strain (1/800) than

for Ardennes- Hargnies strain (animal 1: 1/200 and animal 2: 1/100). The levels of RNA viral loads in sera had already decreased in all four animals analyzed but they were still positive. We also analyzed RNA viral loads in lung and liver, *i.e.* organs where PUUV antigen was previously detected (10, 11). We found high levels of viral RNA loads (**Table 2**), what confirmed the effective infection of all animals by PUUV.

Molecular analyses were carried out as already described (12) to assess PUUV evolution during bank vole infection. RNA was extracted from lung tissue samples of positive bank voles (natural populations and experimentally infected) and from supernatant of Vero E6 cells, then sequenced to obtain the complete coding sequence of the S and M segments. RNA was amplified using RT-PCR and nested PCR as already described (4). PCR products were sequenced using Sanger method. The S and M sequences from Ardennes - Hargnies and Loiret - Vouzon were aligned using the Clustal Omega alignment program implemented in Seaview 4.5.0 (13). Nucleotide sequences were translated into amino acid sequences and analyzed using SeaView 4.5.0. For the M segment (3444 bp for coding region), the PUUV nucleotidic sequences from Ardennes – Hargnies and Loiret - Vouzon were 100% identical when comparing sequences obtained from tissue or supernatant. For the S segment (1304 bp for coding region), the amino acid sequences from Ardennes – Hargnies and Loiret - Vouzon revealed one mismatch between the sequences gathered from the tissue and the supernatant (**Figure 2**). Therefore, isolation protocols (*in vitro* or *in vivo*) seemed to have influenced the amino acid sequences of both PUUV strains.

We next performed high throughput sequencing analyses to deeper investigate these genetic variations. More specifically, we aimed at assessing whether the different isolation protocols maintained the initial intra-host viral diversity detected in the original samples. We thus characterized the viral quasispecies pool at each step of the different isolation processes. PUUV

S segment (1750bp) was sequenced using MiSeq Illumina technology with 10 overlapping amplicons (named A to J) of about 250 bp (see supporting information S1). 300ng of viral RNA from supernatant of Vero cells and 1500ng from lung tissue of infected rodents were reverse transcribed with SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. The reverse transcription reaction was performed with 2µM of primers PUU1F1 (5'-CCTTGAAAAGCTACTACGAG-3') and PUU1R1 (5'-CCTTGAAAAGCAATCAAGAA-3') and with 50ng of random hexamers provided in the kit. The sequencing libraries were prepared using a two-step PCR strategy adapted from Galan et al. 2018 (14) (see supporting information S2) and combined with a unique dual indexes (UDI) multiplex sequencing approach (15): each 9-bp i5 and i7 dual index were used only for one PCR sample without combinatorial indexing, to make sure that libraries were sequence and demultiplex with the highest accuracy without problem of “leak” between libraries (16). The libraries were sequenced on a MiSeq platform (GenSeq, Montpellier, France) with a 500-cycle reagent kit v2 (Illumina). A run of 250bp paired-end sequencing was performed. The sequences were analysed with the Data pre-processing tool of FROGS (Genotoul) (17) and the chimeric variants were remove using isBimeraDenovo function of Dada2 R package (18). Each sample was analysed independently (qRT-PCRs, PCR amplifications and sequencing) using at least three PCR replicates to distinguish the true genetic variants and the artefactual mutations due to polymerase or sequencing errors. Mutations below a threshold of 0.32% were removed (see supporting information S3). Validated variants were aligned and analysed using SeaView 5.0 (13). Two measures were used to compare the viral diversity between the two isolation protocols and the two strains: the number of polymorphic sites (19, 20) and the mean of percent complexity (the number of unique sequence reads/total reads × 100) calculated on the 10 amplicons (21). Kruskall-Wallis test followed by Dunn multiple comparison test were

conducted to compare the mean of percent complexity between strains origin (in natura, in vitro or in vivo).

In natura, the viral diversity between Ardennes - Hargnies and Loiret - Vouzon strains were similar. We found no difference in the number of polymorphic sites or in the mean of percent complexity between areas. After the *in vitro* isolation process, one variant of Ardennes - Hargnies strain (R63), mostly found *in natura*, and was still present at high frequency (about 23%) instead of a switch in major variant as Q63. The viral diversity decreased after the cell culture passages, whatever the diversity index considered. For Loiret - Vouzon strain, as observed with Sanger method we can see a switch of major variant to from A28 to S28. But HTS results were contradictory: the number of polymorphic sites increase but the mean of percent complexity stay stable. The *in vitro* isolation process lead to a significant difference of viral diversity between PUUV strains but not in the same way for both strains **(Figure 3A)**.

After the *in vivo* isolation process, we detected a lower number of polymorphic sites compared to natural strains. However we found no significant difference of mean of percent complexity when comparing PUUV strains or *in natura* and *in vitro* conditions **(Figure 3B)**. The sequence of the majority variant did not differ between these two conditions, whatever the isolation protocol considered. Results were similar between bank voles infected with the same strain.

Conclusion

In this study, we were able to cultivate and maintain in cell culture, for the first time, two PUUV isolates from two distinct French areas. Molecular analyses of the S and M segments of PUUV originating from natural isolates, bank voles experimentally infected and cell cultures revealed only one amino-acid mismatch for Loiret - Vouzon and Ardennes – Hargnies strains. Both mismatch were identified in cell culture. High throughpout sequencing confirmed this result, enabling to define a “bank vole” and a “cell culture” signature. Having these two modes of

PUUV wild strain culture is an important asset. Cell adapted strains allow to dispose of well-characterized viruses that can be used for antiviral candidate studies or cell experimentations assessing the role of apoptosis, PUUV propagation or control (22). Besides, wild strains maintained in their natural host will better contribute to improve our knowledge of PUUV ecology and evolution (23). In *in vivo* experiment, bank vole used came from "neutral" origin: not from Ardennes or Loiret. For the moment, bank vole-PUUV interactions were poorly documented but could be a key point in viral diversity.

This study also provides new arguments enabling to understand the regional epidemiological differences concerning the circulation of these PUUV strains in humans and in bank voles (4). It has previously been shown that there is a significant inter-regional viral genetic diversity (4, 5, 24), that could explain, at least partly, the regional differences in NE epidemiological status in France. Here, we showed that viral diversity of PUUV circulating in the NE endemic area (Ardennes) and in the NE non-endemic area (Loiret) evolved differently when it passaged on VeroE6 cells. Further research dedicating to the characterization of the French Puumala strains now available will help deepening our knowledge on the NE epidemiological situation.

Ethics statement: All animal studies were conducted in accordance with French and European regulations on the care and protection of laboratory animals (French Law 2013-118 from February 1st, 2013 and Directive 2010/63/EU from September 22, 2010).

Acknowledgments: Data used in this work were partly produced through sequencing facilities of ISEM (Institut des Sciences et de l'Evolution-Montpellier) and LabEx CEMEB (Centre Mediteraneen Environnement Biodiversité) Sarah Madrières was funded by an INRA-EFPA/ANSES fellowship.

References

1. Lee HW, Vaheri A, Schmaljohn CS. Discovery of hantaviruses and of the Hantavirus genus: personal and historical perspectives of the Presidents of the International Society of Hantaviruses. Virus Res. 2014 Jul 17;187:2-5.
2. Brummer-Korvenkontio M, Vaheri A, Hovi T, von Bonsdorff CH, Vuorimies J, Manni T, et al. Nephropathia epidemica: detection of antigen in bank voles and serologic diagnosis of human infection. J Infect Dis. 1980 Feb;141(2):131-4.
3. Jean-Marc Reynes DC, Béatrice Renaudin, Alexandra Fizet, Jean-Baptiste Bour, Véronique Brodard, Emmanuelle Cart-Tanneur, Annie Dewilde, Mohamed El Hamri, Hervé Fleury, Denise Hecquet, Hélène Jeulin, Sylvie Lechat, Maud Lemarquand-Bardini, Quentin Lepiller, Jean-Dominique Poveda, Olivia Raulin, Eterne Twizeyimana, Didier Van Cauteren, Aurélie Velay, Alexandra Septfons, Sylvain Baize. Surveillance of human hantavirus infections in metropolitan France, 2012-2016. Bulletin Epidémiologique Hebdomadaire. 2017;23:492-9.
4. Castel G, Couteaudier M, Sauvage F, Pons JB, Murri S, Plyusnina A, et al. Complete Genome and Phylogeny of Puumala Hantavirus Isolates Circulating in France. Viruses. 2015 Oct 22;7(10):5476-88.
5. Reynes JM, Carli D, Thomas D, Castel G. Puumala Hantavirus Genotypes in Humans, France, 2012-2016. Emerg Infect Dis. 2019 Jan;25(1):140-3.
6. Lundkvist A, Cheng Y, Sjolander KB, Niklasson B, Vaheri A, Plyusnin A. Cell culture adaptation of Puumala hantavirus changes the infectivity for its natural reservoir, Clethrionomys glareolus, and leads to accumulation of mutants with altered genomic RNA S segment. J Virol. 1997 Dec;71(12):9515-23.
7. Seto T, Tkachenko EA, Morozov VG, Tanikawa Y, Kolominov SI, Belov SN, et al. An efficient in vivo method for the isolation of Puumala virus in Syrian hamsters and the characterization of the isolates from Russia. J Virol Methods. 2011 Apr;173(1):17-23.
8. Klingstrom J, Heyman P, Escutenaire S, Sjolander KB, De Jaegere F, Henttonen H, et al. Rodent host specificity of European hantaviruses: evidence of Puumala virus interspecific spillover. J Med Virol. 2002 Dec;68(4):581-8.
9. Sundstrom KB, Stoltz M, Lagerqvist N, Lundkvist A, Nemirov K, Klingstrom J. Characterization of two substrains of Puumala virus that show phenotypes that are different from each other and from the original strain. J Virol. 2011 Feb;85(4):1747-56.
10. Gavrilovskaya IN, Apekina NS, Myasnikov Yu A, Bernshtein AD, Ryltseva EV, Gorbachkova EA, et al. Features of circulation of hemorrhagic fever with renal syndrome (HFRS) virus among small mammals in the European U.S.S.R. Arch Virol. 1983;75(4):313-6.
11. Yanagihara R, Amyx HL, Gajdusek DC. Experimental infection with Puumala virus, the etiologic agent of nephropathia epidemica, in bank voles (Clethrionomys glareolus). Journal of Virology. 1985;55(1):34-8.
12. Binder F, Reiche S, Roman-Sosa G, Saathoff M, Ryll R, Trimpert J, et al. Isolation and characterization of new Puumala orthohantavirus strains from Germany. Virus Genes. 2020 Aug;56(4):448-60.
13. Gouy M, Guindon S, Gascuel O. SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol Biol Evol. 2010 Feb;27(2):221-4.

14. Galan M, Pons JB, Tournayre O, Pierre E, Leuchtmann M, Pontier D, et al. Metabarcoding for the parallel identification of several hundred predators and their prey: Application to bat species diet analysis. Mol Ecol Resour. 2018 May;18(3):474-89.
15. Kircher M, Sawyer S, Meyer M. Double indexing overcomes inaccuracies in multiplex sequencing on the Illumina platform. Nucleic Acids Res. 2012 Jan;40(1):e3.
16. Martin J-F. Creating error-proof indexes for high throughput sequencing. Zenodo. 2019.
17. Escudié F, Auer L, Bernard M, Mariadassou M, Cauquil L, Vidal K, et al. FROGS: Find, Rapidly, OTUs with Galaxy Solution. Bioinformatics. 2017;34(8):1287-94.
18. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016 Jul;13(7):581-3.
19. Gregori J, Perales C, Rodriguez-Frias F, Esteban JI, Quer J, Domingo E. Viral quasispecies complexity measures. Virology. 2016 Jun;493:227-37.
20. Zhao L, Illingworth CJR. Measurements of intrahost viral diversity require an unbiased diversity metric. Virus Evolution. 2019;5(1).
21. Cousins MM, Ou SS, Wawer MJ, Munshaw S, Swan D, Magaret CA, et al. Comparison of a high-resolution melting assay to next-generation sequencing for analysis of HIV diversity. J Clin Microbiol. 2012 Sep;50(9):3054-9.
22. Sola-Riera C, Gupta S, Ljunggren HG, Klingstrom J. Orthohantaviruses belonging to three phylogroups all inhibit apoptosis in infected target cells. Sci Rep. 2019 Jan 29;9(1):834.
23. Madrieres S, Castel G, Murri S, Vulin J, Marianneau P, Charbonnel N. The Needs for Developing Experiments on Reservoirs in Hantavirus Research: Accomplishments, Challenges and Promises for the Future. Viruses. 2019 Jul 19;11(7).
24. Monchatre-Leroy E, Murri S, Castel G, Calavas D, Boue F, Henaux V, et al. First insights into Puumala orthohantavirus circulation in a rodent population in Alsace, France. Zoonoses Public Health. 2018 Aug;65(5):540-51.

Figure and table legend

<u>Figure 1:</u> PUUV isolation with Vero E6 cells.

Kinetics of PUUV RNA quantity during isolation process (**A**). Circles represent supernatants sampling. Picture of viral titration results (**B**) for Hargnies and Vouzon compared to Sotkamo referential strain.

<u>Figure 2:</u> Comparison of S and M segment sequences.

Synthesis of Sangers sequencing of S and M segments for Natural Isolates (sequence as reference), cell cultivated viruses and experimentally infected bank voles.

Bank vole and cells drawing were designed by brgfx / Freepik

Figure 3:

Comparison of viral diversity after (**A**) *in vitro* and (**B**) *in vivo* isolation process.

Table 1: Serological results.

Serological results of bank vole experimentally infected with lung homogenates from animals trapped from Hargnies and Vouzon.

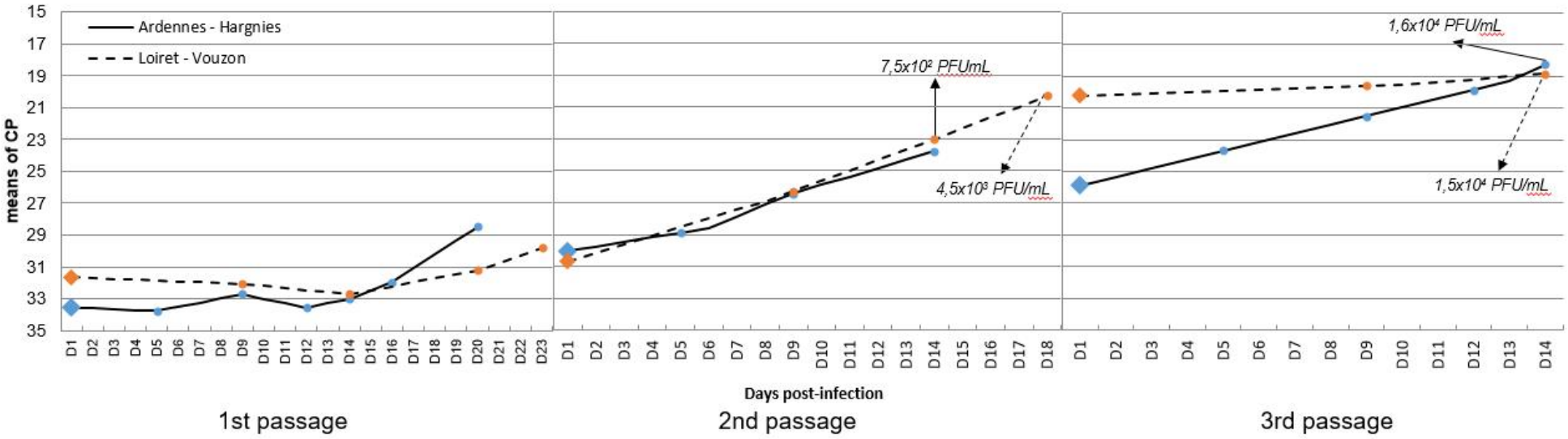
OD : Optical Density – ND : Not Defined

Table 2: RNA detection results.

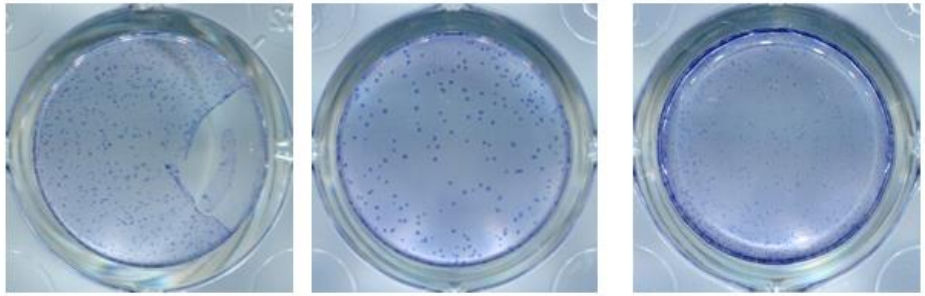
RNA detection results of bank vole experimentally infected with lung homogenates from trapped animals at Hargnies and Vouzon.

Figure 1

A



B



Hargnies isolate | *Positive Control – Strain Sotkamo* | *Vouzon isolate*

Table 1

Capture area	Day post infection	animal number	Antibody IgG-NPuu IgG-ELISA OD	Conclusion	Titration Last + dilution (OD)
Vouzon	**J7**	791	0 ,032	-	ND
	J7	792	0,029	-	ND
	J9	791	0,23	+	800 (0,105)
	J9	792	0,227	+	800 (0,106)
Hargnies	**J7**	811	0,09	-	ND
	J7	812	0,058	-	ND
	J9	811	0,14	+	200 (0,102)
	J9	812	0,117	+	100 (0,104)

Table 2

Strain	Day post infection	animal number	RT-PCR (mean CP)			
			Lung	Liver	Sera - Day7	Sera – Day 9
Vouzon	J9	791	19,0	21,0	30,7	31,0
		792	19,1	20,8	29,9	30,5
Hargnies	J9	811	16,5	19,7	29,2	32,1
		812	19,1	20,0	30,6	32,7

Figure 2

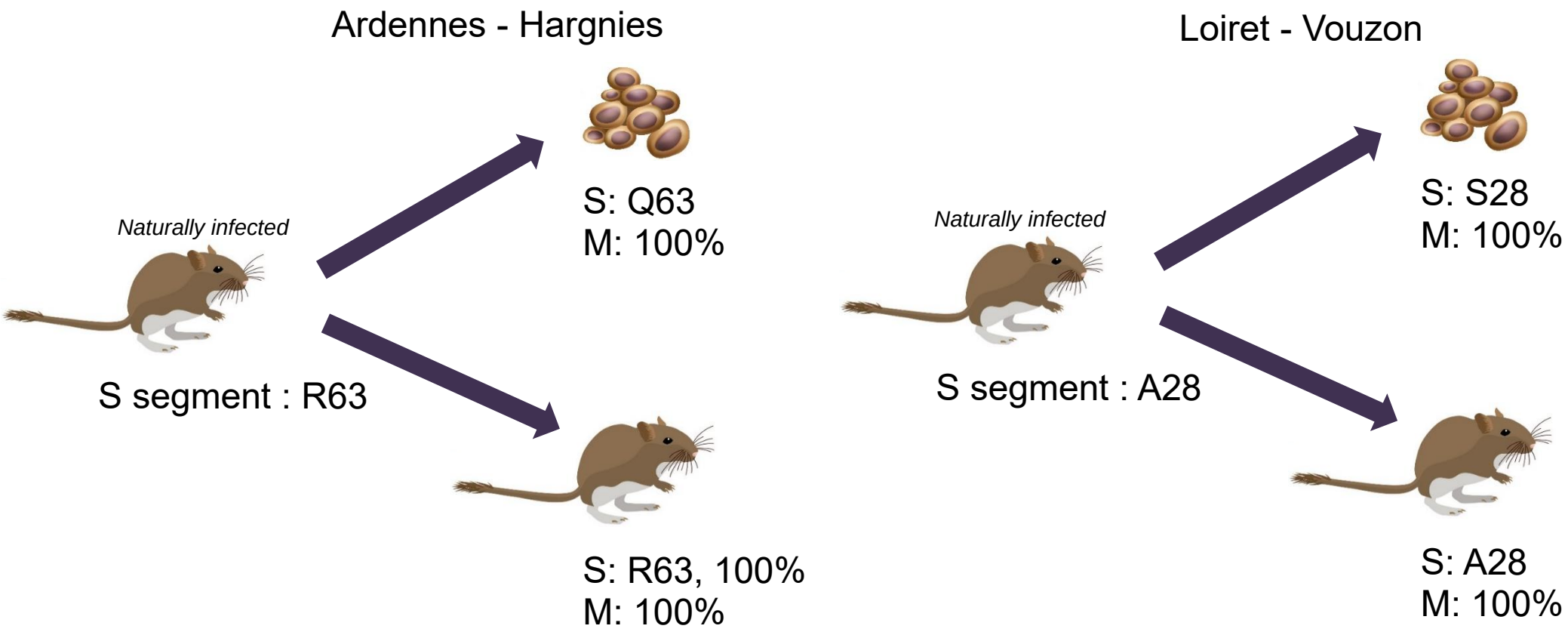
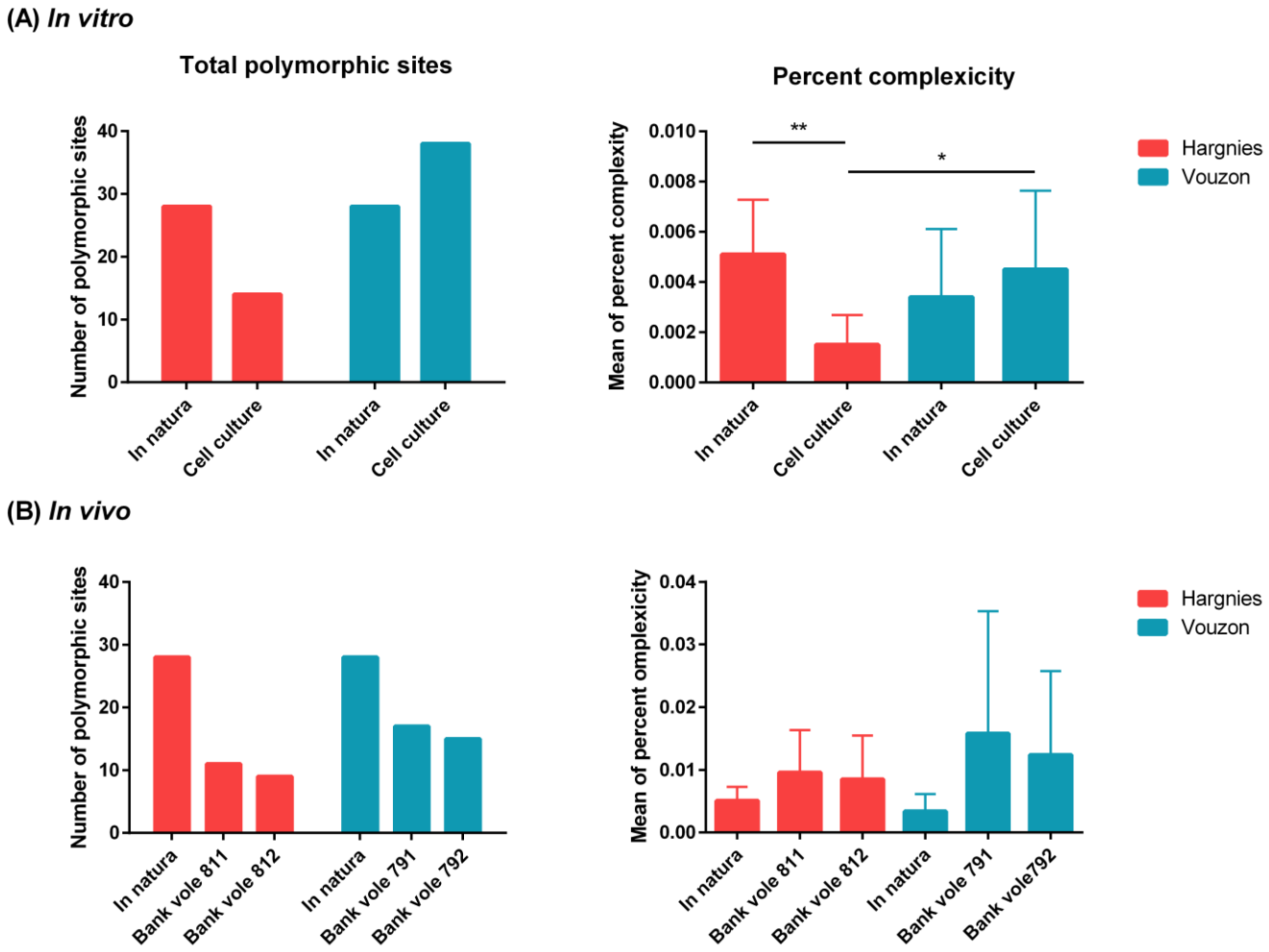

Ardennes - Hargnies
Naturally infected
S segment : R63
S: Q63
M: 100%
S: R63, 100%
M: 100%
Loiret - Vouzon
Naturally infected
S segment : A28
S: S28
M: 100%
S: A28
M: 100%

Figure 3


(A) In vitro
Total polymorphic sites
Number of polymorphic sites
40
30
20
10
0
In natura
Cell culture
In natura
Cell culture
Percent complexicity
Mean of percent complexity
0.010
0.008
0.006
0.004
0.002
0.000
**
*
In natura
Cell culture
In natura
Cell culture
Hargnies
Vouzon
(B) In vivo
Number of polymorphic sites
40
30
20
10
0
In natura
Bank vole 811
Bank vole 812
In natura
Bank vole 791
Bank vole 792
Mean of percent omplexicity
0.04
0.03
0.02
0.01
0.00
In natura
Bank vole 811
Bank vole 812
In natura
Bank vole 791
Bank vole792
Hargnies
Vouzon