Journal Pre-proof

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PII: S0166-0934(19)30208-3

DOI: https://doi.org/10.1016/j.jviromet.2019.113729

Reference: VIRMET 113729

To appear in: *Journal of Virological Methods*

Received Date: 9 May 2019

Revised Date: 15 August 2019

Accepted Date: 7 September 2019

Please cite this article as: Binder F, Lenk M, Weber S, Stoek F, Dill V, Reiche S, Riebe R, Wernike K, Hoffmann D, Ziegler U, Adler H, Essbauer S, Ulrich RG, Common vole (*Microtus arvalis*) and bank vole (*Myodes glareolus*) derived permanent cell lines differ in their susceptibility and replication kinetics of animal and zoonotic viruses, *Journal of Virological Methods* (2019), doi: https://doi.org/10.1016/j.jviromet.2019.113729

Common vole (*Microtus arvalis*) and bank vole (*Myodes glareolus*) derived permanent cell lines differ in their susceptibility and replication kinetics of animal and zoonotic viruses

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Highlights

- Development of cell lines of the bank vole and the common vole, two important pathogen reservoirs
- Establishment of potent host cell lines for the propagation of Puumala and Tula orthohantavirus
- Productive replication of several viruses in the vole cells
- Vole species-associated differences in the susceptibility of the cell lines

- Tissue-specific differences in the susceptibility for various viruses
- Useful tools to characterize host cell factors involved in virus replication

Abstract

Pathogenesis and reservoir host adaptation of animal and zoonotic viruses are poorly understood due to missing adequate cell culture and animal models. The bank vole (*Myodes glareolus*) and common vole (*Microtus arvalis*) serve as hosts for a variety of zoonotic pathogens. For a better understanding of virus association to a putative animal host, we generated two novel cell lines from bank voles of different evolutionary lineages and two common vole cell lines and assayed their susceptibility, replication and cytopathogenic effect (CPE) formation for rodent-borne, suspected to be rodent-associated or viruses with no obvious rodent association. Already established bank vole cell line BVK168, used as control, was susceptible to almost all viruses tested and efficiently produced infectious virus for almost all of them. The Puumala orthohantavirus strain Vranica/Hällnäs showed efficient replication in a new bank vole kidney cell line, but not in the other four bank and common vole cell lines. Tula orthohantavirus replicated in the kidney cell line of common voles, but was hampered in its replication in the other cell lines. Several zoonotic viruses, such as Cowpox virus, Vaccinia virus, Rift Valley fever virus, and Encephalomyocarditis virus 1 replicated in all cell lines with CPE formation. West Nile virus, Usutu virus, Sindbis virus and Tick-borne encephalitis virus replicated only in a part of the cell lines, perhaps indicating cell line specific factors involved in replication. Rodent specific viruses differed in their replication potential: Murine gammaherpesvirus-68 replicated in the four tested vole cell lines, whereas murine norovirus failed to infect almost all cell lines. Schmallenberg

virus and Foot and mouth disease virus replicated in some of the cell lines, although these viruses have never been associated to rodents. In conclusion, these newly developed cell lines may represent useful tools to study virus-cell interactions and to identify and characterize host cell factors involved in replication of rodent associated viruses.

Keywords:

Cell line; Cytopathogenic effect; Host; Vole

1. Introduction

Emerging viruses play an important role in animal and human health and zoonotic agents cause a variety of human infections in the world (1). New high-throughput methods allowed the rapid identification of novel animal or zoonotic pathogens (2). Small mammals and rodents in particular represent a substantial part of the worldwide mammal diversity and have an important impact on human civilization (3). In addition to their role as pests in agriculture and forestry they are frequently competing with human and domestic animal food resources. Most importantly, they are associated with numerous zoonotic agents, such as orthohanta-, arena- and orthopoxviruses, *Leptospira* spp. or a variety of endoparasites (4). Due to their wide distribution, high abundance and close proximity to humans or farm, companion and pet animals, human infections with rodent-borne pathogens are frequently observed.

Bank vole (*Myodes glareolus*) and common vole (*Microtus arvalis*) harbor a variety of zoonotic agents, e.g. orthohantaviruses (5; see Table 1). Tula orthohantavirus (TULV) is frequently detected in the common vole, but also in related *Microtus* and other vole species (6, 7). Large-scale sequence analyses of TULV strains indicated well-separated genetic clades with defined geographical distribution, e.g. Moravia strain belongs to the Eastern South (EST.S) clade in the Eastern evolutionary lineage of the common vole (8, 9). The zoonotic potential of TULV is controversially discussed (10). Puumala orthohantavirus (PUUV) in contrast is the main causative agent of hemorrhagic fever with renal syndrome (HFRS) in Europe (10). It is excreted by the bank vole in feces, urine, and saliva and is transmitted *via* inhalation of aerosols or by biting. PUUV in Germany and its neighboring countries in the west is associated with the Western evolutionary lineage of the bank vole (11). Sympatrically occurring bank voles of the Eastern lineage or the Carpathian lineage seem to be susceptible for PUUV carried by the Western lineage in Germany (11). PUUV and TULV are difficult to grow in cell culture as they need long incubation times and replicate only to low titers (12).

Cowpox virus (CPXV), family *Poxviridae*, is another zoonotic virus associated with rodents, and common and bank vole in particular, as reservoirs (13, 14). Vaccinia virus (VACV) is a further zoonotic orthopox virus that is detected in rodents in South America (15, 16, Table 1). The encephalomyocarditis virus (EMCV) has a worldwide distribution and can infect a broad spectrum of mammal species, but its natural reservoir host is believed to be a rodent. It was described to be a zoonotic agent, but an association between human infection and disease has still not been clearly established (17).

Rodents also play a pivotal role in the infection cycle of many important arthropod-borne viruses, such as tick-borne encephalitis virus (TBEV). This virus was detected

in wild and experimentally infected common and bank voles (18). Voles are discussed to serve as a reservoir on which ticks and their larvae and nymphs get TBEV infected during blood-feeding or co-feeding (19). Usutu virus (USUV) and Sindbis virus (SINV) affect birds, but were also detected in rodents and discussed to have zoonotic potential (20, 21). Rift Valley Fever virus (RVFV) is another vector-borne zoonotic pathogen that was detected in rodents (Table 1). West nile virus (WNV) is transmitted by *Culex* mosquitoes and was repeatedly introduced into Europe, especially Italy, Greece, Romania (22), and recently Germany (23). The WNV replication cycle involves mosquito species as vector and wild birds acting as the reservoir, but with spillover to humans, rodents and other mammals (24). Currently, there are not many studies exploring the WNV host range. In contrast, Schmallenberg virus (SBV) is a vector borne, non-zoonotic pathogen that has not been detected in rodents before (25; see Table 1).

Murine norovirus (MNV) and Murine gammaherpesvirus 68 (MHV-68) are rodent-specific model viruses used for studies on noro- and herpesviruses (26). MHV-68 was isolated from rodents and showed a high prevalence in yellow necked mouse (*Apodemus flavicollis)* and bank vole (27). Foot and mouth disease virus (FMDV) is a picornavirus that affects livestock worldwide. It can infect a variety of hosts with cloven-hoofed animals playing a central role in its cycle, however it was never detected in rodents (28, 29; see Table 1).

Currently, many viruses, including those associated to rodents, are isolated and propagated in standard cell lines from African green monkeys (Vero cells), baby hamster kidney cells (BHK-21) or mosquito cells (clone C6/36) (30). The Vero cell subclone E6 provides an excellent environment for viruses to replicate as it cannot provide a functional type-I interferon response (31). Vero E6 cells have enabled the

isolation of a variety of hantaviruses, however most novel rodent-borne viruses remain uncultured (32-37). Rodent-derived cell culture models, which reflect the unique virus-host adaptation, are still rare. They can be beneficial for virus isolation and characterization of host specificity and identification of cellular receptors or host cell factors that are essential for development of strategies counteracting these pathogens (2). Previously the characterization of a permanent bank vole-derived kidney cell line (BVK168) for propagation of different viruses was described (38). However, mainly primary bank vole cells were used for propagation of hantaviruses so far (39, 40). Primary cell cultures are tissue derived cells that were freshly isolated and have a limited live span *in vitro*. They mostly keep their *in vivo* properties and therefore are often used for studying cellular processes and gene-expression. However, as they derive from blood or organ tissue, the operator faces challenges like fragile handling processes or higher risk due to unknown infectious status. Additionally, experiments depend on the availability of fresh organ material for cell extraction. Permanent cell culture systems on the other hand are immortalized cells or tumor cells, which are available on request as their lifespan is not limited. The drawback of these cells is that they lose many of their natural properties as they undergo mutations in the genome or accumulate chromosome multiplications. These are valuable for research as storage by cryo-conservation is possible, which allows standardized long-term experiments. This also leads to less dependency on certain growth factors associated with easier handling (41).

Herein we describe the establishment of four novel cell lines: the common vole kidney cell line FMN-R (FMN), the common vole brain cell line FMG-R (FMG), the Eastern lineage bank vole lung cell line MGLU-2-R (MGLU) and the Carpathian lineage bank vole kidney cell line MGN-2-R (MGN). We have chosen 14 laboratory adapted viruses

out of nine virus families that are either rodent-borne and zoonotic, suspected to be rodent-associated or have no obvious association to rodents (Table 1). The replication and cytopathic effect (CPE) formation in FMN, FMG and MGLU cell lines and the previously described Western lineage bank vole-derived BVK168 kidney cell line (38) were compared. The MGN cell line was used here exclusively for infection studies of PUUV as the previously described BVK168 cell line was not able to efficiently replicate PUUV.

2. Materials and Methods

2.1 Cell lines

The generation and characterization of the cell line BVK168 (Collection of Cell Lines in Veterinary Medicine – Riems (CCLV-RIE) 1313)) was described previously to be of epitheloid morphology and male origin (38).

To generate the MGN cell line (CCLV-RIE 1494), an adult bank vole was obtained from the holding of the Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany. The animal was anaesthetized with isofluran and euthanized by using CO_2. The kidney of the bank vole was extracted and minced. Cultures were generated by explant culture method in an equal mixture of Ham's F12 and Iscove's modified Dulbecco's medium

(IMDM) containing 10% fetal calf serum (FCS). Resulting cells were subcultured to passage 12 and determined to be of epithelial-like morphology. PCR-typing revealed a male sex of the cell line.

To generate the MGLU cell line (CCLV-RIE 1304), an adult female bank vole was live trapped on the island of Riems, Greifswald, Germany (permission of the trapping: Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern 7221. 3-030/09). The animal was anaesthetized with isofluran and euthanized by using CO_2. During dissection the whole right lung (*pulmo dexter*) was taken, fractionated and trypsinized three times for 30 min at room temperature. The resulting cells were seeded in a mixture of equal volumes of minimal essential medium (MEM) with Earle's balanced salt solution (BSS) and MEM (Hanks' BSS), containing 10% fetal calf serum. The cells were propagated continuously in a closed system to subculture no. 54 showing an increase in proliferation around subculture no. 25 (altered splitting ratio from 1:2 to 1:6 to 1:10), where we suppose that spontaneous immortalization occurred. Cells were determined as fibroblast-like and of female sex by PCR typing.

For generation of novel common vole cell lines FMN (CCLV-RIE 1102) and FMG (CCLV-RIE 1129), common voles were obtained from the holding of the Julius Kühn-Institute, Münster, Germany. To generate FMN, kidneys of six newborn common voles (4-6 days old) were minced together. After fractionated trypsination three times for 30 min at room temperature, the cells were seeded in MEM including Hanks' BSS and 10% FCS. After changing the medium to a mixture of equal volumes of Ham's F12 and Iscove's modified Dulbecco's medium containing 20% FCS, the cells were subcultured in a closed system without any sign of crisis to subculture no. 70. Spontaneous immortalization seemed to occur around subculture no. 20, when the splitting ratio

could be increased from 1:2 to 1:4. Cells at this passage demonstrated a fibroblastoid morphology.

To generate FMG, brains of six newborn common voles were pooled, minced with scissors and a syringe with a needle. The cells were seeded in MEM (Hanks' BSS) containing 10% FCS. After changing the medium to a mixture of equal volumes of Hams' F12 and IMDM with 20% FCS, the cells showed better proliferation. The fibroblastoid cells were subcultured in a closed system continuously without any crisis until subculture no. 100. Spontaneous immortalization seemed to happen around passage no. 40.

All viruses and cell lines were tested free of bacterial contamination by mycoplasma PCR screening and standard in-house bacterial isolation approaches. Analysis of potential contaminations for all cell lines was further investigated by the Multiplex cell Contamination Test (McCT) Service, Heidelberg, Germany (http://www.multiplexion.de). This test includes multiplex PCR assays for 14 *Mycoplasma* species, Squirrel monkey retrovirus, and Epstein-Barr virus. In addition, potential contamination with human, *Macaca cynomolgus*, mouse, rat, Chinese hamster, Syrian hamster, feline, canine, rabbit, pig, Guinea pig and *Drosophila* cells was excluded.

Cells were regularly grown in MEM containing non-essential amino acids and 10% FCS. MGN cell line was grown in Ham's F12/IMDM + 10% FCS. Passaging was done twice a week by trypsinating cells and seeding in a ratio of 1:4 for BVK168, 1:10 for MGLU, 1:6 for MGN, 1:3 for FMN, and 1:20 for FMG.

2.2 Determination of the species, evolutionary lineage origin and sex of the cell lines

The species and evolutionary lineage origin of the cell lines were determined by PCR-based determination of a partial sequence of the mitochondrial cytochrome *b* gene as described previously (11, 42). Cell culture samples were directly used for DNA extraction with a GeneMATRIX Tissue DNA Purification Kit (Roboklon, Potsdam, Germany). Sequences of the cytochrome *b* gene were deposited in GenBank with accession numbers FJ528598 (BVK168), MK559348 (MGLU), MK559347 (MGN), MK559346 (FMN) and MK559345 (FMG) (Supplementary Figure 1). Molecular sex determination was done as described previously using a PCR targeting the Sry and ZFX genes (43).

2.3 Virus inoculation and CPE detection

For analysis of the susceptibility and virus replication in the newly established cell lines and the BVK168 cell line we used 14 laboratory adapted viruses of different taxa and host association (Table 1). To evaluate virus propagation in the vole cell lines, a positive control infection was done in parallel on the specific reference cell line for each virus (see Figure 1B, Table 1). Viruses were inoculated with a multiplicity of infection (MOI) of 0.1 in 1ml serum free Leibovitz (L)-EM medium to 24 hours old cell-monolayers in 12.5cm² flasks. After adsorption for 1h at 37°C, 4ml L-EM containing 2% FCS was added and cells were observed daily until a prominent CPE formation was observed. CPE formation was documented by imaging with a Nikon Eclipse TS100

microscope coupled to a Nikon Digital Sight DS-L3 imaging system. Flasks of infected cells were frozen after prominent CPE (60-80%) was obtained or ultimately 7 days post inoculation (d.p.i.). For CPXV total cell sediments were collected in Tris-HCl pH 8 buffer and lysates were used for further investigations.

The protocol was modified for TULV and PUUV infection, as these viruses do not induce a visible CPE (44). FMN, FMG, MGLU and BVK168 cell lines were inoculated with TULV strain Moravia (35) or PUUV strain Vranica/Hällnäs with a MOI of 0.1 for 2h in 0.5ml MEM containing 5% FCS in a 6-well plate. After adsorption, 1.5ml MEM with 5% FCS were added and cells were incubated at 37°C for up to 10 days. PUUV infection was tested at day 10 by immunofluorescence staining with nucleocapsid (N)-protein specific monoclonal antibody 5E11 (45) and an Alexa fluor 488 labelled secondary anti-mouse antibody (Abcam, Cambridge, UK). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific, Waltham, MA, USA). For TULV, infected cells were harvested at several time points, lysed in 2x SDS-PAGE sample buffer (0.5M Tris pH 6.8, 25% glycerin, 10% SDS and 0.5% bromphenolblue) and subjected to Western blot analysis with N-protein specific monoclonal antibody TULV1 diluted 1:10 in PBS-Tween 0.05% (Spakova, Koellner, Ulrich et al., unpubl. data) and a horse radish peroxidase (HRP) labelled secondary goat anti-mouse IgG antibody diluted 1:3000 in PBS-Tween 0.05% (Bio-Rad, Hercules, CA, USA). Alternatively, FMN and BVK168 cells were incubated with TULV strain Moravia for 2h, washed 3 times and kept at 37°C for 18 days until supernatant was collected and frozen at -80°C. Subsequently, Vero E6 reference cells and FMN or BVK168 cells were incubated with supernatants of infected cells. Infection was evaluated after 10 days by immunostaining of hantaviral N protein using antibody TULV1 and an Alexa-Fluor 488 labelled secondary anti-mouse antibody (Abcam). Nuclei were stained with DAPI.

$2x10^5$ cells of the MGN cell line were seeded in 6-well plates one day before inoculated with PUUV strain Vranica/Hällnäs at MOI of 0.1 or 0.001 for 2h in 0.5ml Ham's F12/IMDM containing 5% FCS. After inoculation, cells were washed 3 times with Ham's F12/IMDM containing 5% FCS and incubated at 37°C for up to 14 days. Every two days one well of cells was fixed and stained with N-protein specific monoclonal antibody 5E11 (45) and an Alexa fluor 488 labelled secondary anti-mouse antibody (Abcam). Nuclei were stained with DAPI as described above. For investigation of infectious particles released from MGN cells, supernatant was taken at day 10 from MOI 0.1 infected cells. As an infection control Vero E6 reference cells were infected in parallel. Fresh naive 24h old monolayers of MGN cells and Vero E6 cells were incubated with the supernatant from MGN cells. Virus propagation was monitored at day 10 by immunostaining of hantaviral N protein as described above.

2.4 Virus titration

Virus titration of cell supernatants/lysates was done in parallel on the original reference cell line for each virus (Table 1, Figure 1B) and the vole cell line used (Figure 1A). After one freeze-thaw cycle, the virus supernatants were serially diluted from 10^{-1} to 10^{-11} in L-EM containing 2% FCS in a 96-well plate with four replicates each. For CPXV two independent experiments with 8 replicates each were performed. Cell monolayers of vole cells or the corresponding reference cell line (see Table 1) were seeded in a 96-well plate one day before. A volume of 100µl of each virus dilution was added to the confluent cells. After incubation for three (FMDV, CPXV, MNV), six (USUV, SINV, RVFV, VACV, SBV, EMCV) or seven (WNV, TBEV, MHV-68) days the 50% tissue

culture infectious dose ($TCID_{50}$) was calculated using the Spearman/Kärber method based on the CPE detection on the cells (46). Virus titers are shown as means with standard deviations. For PUUV and TULV titration of day 10 supernatants, infection of the dilutions was evaluated after 10 days using the immunofluorescence test protocol described above.

For titration of PUUV, MGN and Vero E6 reference cells were infected with MOI 0.1 and 0.001 as described in detail in chapter 2.3. Supernatants of both cell lines were collected every two days and frozen at -80°C. Subsequently, supernatants were serially diluted from 10^{-1} to 10^{-7} in MEM containing 5% FCS in a 96-well plate with three replicates each. Vero E6 cells were seeded in 96 well plates one day before, and a volume of 100µl of each virus dilution was added to these cell monolayers for virus titration. After incubation for 10 days, the $TCID_{50}$ was calculated using indirect immunofluorescence for PUUV N protein detection as described. Titers were calculated by the Spearman/Kärber method and mean titers of three experiments are given with standard deviation (SD).

3. Results

3.1 BVK168 cell line is highly susceptible and promotes replication of almost all viruses investigated

The BVK168 cell line was determined to originate from a bank vole of the Western evolutionary lineage (Supplementary Figure 1). All viruses chosen for the infection resulted in visible CPE and viral propagation, as documented in reference and BVK168 cell lines, except for MNV (Figure 2A, Figure 3A, Tables 2 and 3A). Hantaviruses as non-cytolytic viruses were evaluated separately and are described below.

Endpoint titration showed that CPXV and VACV amplified to similar quite high titers of about 10^5 $TCID_{50}$/ml, independently if back-titrated to reference or BVK168 cells (Fig. 3A). However, the titer in the reference control cell line was about 0.5 logs higher for both orthopox viruses. The kinetics of CPE formation differed between both viruses (Table 3A).

Additional testing of this cell line indicated a high susceptibility for the tick-borne zoonotic TBEV (Table 3A, Figures 2A and 3A). The obtained titer in the back-titration was higher in the bank vole cell line compared to the reference cell line. However, when the original virus was grown on the reference cell line Vero B4 titers were still highest (Figure 3A). Mosquito-borne viruses like SINV, WNV and RVFV replicated in BVK168 cells to titers that differed only slightly in the back-titration between reference cell line and BVK168 cell line. For another mosquito-borne virus, USUV, titers after inoculation of BVK168 cells were about two logs lower when back titrated on BVK168 cells in comparison to the Vero E6 reference cells (Figure 3A). Even higher was the observed titer, when original USUV was grown on the reference Vero E6 cells (Figure 3A). CPE was observed at 2 (SINV) up to 6 (USUV) days p.i. (Table 3A, Figures 2A and 3A).

Inoculation of the BVK168 cell line with non-zoonotic viruses (MNV, FMDV) and with EMCV 2 indicated delayed or no replication and CPE formation if compared to their corresponding reference cell lines (Tables 2 and 3A, Figures 2A and 3A). MHV-68 was demonstrated to behave similar in terms of CPE formation kinetics in BVK168 and reference cells. Virus titers on BVK168 differ only by about one log when back titrated on both cell lines (Figure 3A, Tables 2 and 3A). Interestingly, SBV replicated in the BVK168 cell line similar as on BHK21 reference cells. BVK168 and BHK21 cell lines

were frozen 3 days p.i. due to prominent disruption of the cell monolayer and similar titers of 10^7 $TCID_{50}$/ml were observed on both cells.

3.2 MGLU cell line differs in its susceptibility and support of replication of several animal and zoonotic viruses

As expected due to the trapping site of the bank vole, the novel cell line MGLU originated from a bank vole of the Eastern evolutionary lineage (see Supplementary Figure 1). The MGLU cell line supported the replication of SINV, RVFV, SBV, VACV, CPXV and MHV-68 as evidenced by titration of the supernatants in the vole and the corresponding reference cell lines (Figure 3B, Tables 2 and 3A). For SINV and MHV-68 the titer in the MGLU and the reference cell lines was the same, whereas for RVFV, CPXV, SBV and VACV the titer in the vole cell line was approximately 3 - 1.5 logs reduced compared to the reference cell line. CPXV very rapidly induced a CPE manifested as prominent plaques in MGLU cells (Table 3B and Figure 2B). Similar results were observed for SINV and VACV with only slight variation of the induction of CPE formation (Table 3, Figure 2B).

FMDV, USUV and WNV showed no or for EMCV 2 very little (residual virus) replication in the MGLU cell line when back-titrated to the same cell line or the virus-specific reference cell lines (Figure 3B). The detection of the CPE formation confirmed this obvious difference: None of these four viruses showed a CPE (Table 3B and Figure 2B). The cell line was also susceptible for TBEV infection, but showed only weak CPE at late time points (Table 3B). Virus replication was detected only by back-titration in the reference cell line (Figure 3B) indicating low or inefficient replication without induction of visible CPE. Similar results were observed for EMCV 1.

MNV showed no CPE and very limited replication when back-titrated to the vole cell line (Figure 3B and Table 3). In contrast, RVFV and SBV were able to induce a CPE in the MGLU cell line after longer incubation times. Titers of 10^3 to 10^6 could be determined on both, reference and MGLU cells with prominent CPE (Figures 2B and 3B).

3.3 FMN and FMG cell lines are highly susceptible for CPXV, RVFV and MHV-68, but differ in their susceptibility to EMCV 1 and SINV

The susceptibility of FMN and FMG cell lines for CPXV and MHV-68 did not differ significantly when evaluating CPE and titer (Tables 4C and 4D, Figures 2C and D, Figures 3C and 3D). Similarly, both cell lines were also susceptible for VACV, but differed slightly in the kinetics of CPE formation. For both cell lines the titration in the vole and reference cell lines revealed similar titers that are 5 logs lower than the original ones in the positive controls indicating limitations in replication (Figures 3C and D).

Analyses of SINV and EMCV 1 showed most obvious differences between both cell lines: In the FMN cell line, a rapid CPE was induced and high titers were observed in both titrations, similar to the titers observed in the control experiments. In the FMG cell line no CPE and a low titer was measured only in the back-titration to the reference cell line (Figures 2C and D, Figures 3C and D, and Table 4).

RVFV caused CPE in both cell lines, but with different kinetics (Table 4). Back titration of the vole cell-derived supernatants in the reference cell line resulted in titers almost identical to those observed in the positive control experiment, but reduced titers for FMN or no titer at all in the back-titration to the FMG cell line (Figures 3C and 3D).

WNV caused CPE on the FMN cell line, but only titers in the back-titration on Vero cells were observed (Table 4A, Figure 3C). Both cell lines did not show any signs of virus infection for EMCV 2, FMDV, USUV and TBEV. However, the back-titration of TBEV and USUV supernatants from infected FMN cells on Vero cells indicated moderate titers, suggesting replication without signs of CPE formation (Figures 3C and 3D).

3.4 FMN cells productively replicate TULV, whereas TULV loses infectivity in BVK168 cells

TULV inoculation of FMN cells resulted in antigen expression as evidenced by Western blot analysis (Figure 4B). Re-inoculation of Vero E6 and FMN cells with supernatant from day 18 of initially infected and washed FMN cells showed infection of both cell monolayers after 10 days (Figure 4D, Table 5). About 70% of FMN cells were infected but showed altered morphology (Figures 4D and 4E). Titers observed on FMN cells after 10 days were comparable to the reference infection and reached titers of 10^6 $TCID_{50}$/ml (Figure 3C). TULV inoculation of BVK168 cells resulted in an increasing N protein detection in cells from day 4 to day 10 p.i. (Figure 4A). Re-inoculation of the supernatant from initially infected and washed BVK168 cells to fresh Vero E6 reference cells resulted in detection of hantaviral N-protein after 10 days and virus titers of approximately 10^5 $TCID_{50}$/ml (Figures 4C and 3A). In contrast, BVK168 cells inoculated with the same supernatant did not show any signs of infection (Figures 4C and 4E) suggesting a common vole specific phenotype. In neither the BVK168 nor the reference cells a CPE was observed at any timepoint (Figures 4C and 4D). FMG and MGLU cells could not be infected with TULV (Figures 3B and 3D, Table 5).

3.5 PUUV replicates efficiently in the bank vole MGN cell line, but not in any other cell line

Analysis of the partial mitochondrial cytochrome *b* gene assigned MGN cells to the Carpathian bank vole lineage (Supplementary Figure 1). When inoculated with PUUV, the cells showed accumulation of hantaviral N protein over time. After inoculation with MOI 0.001, infection of the cells could be observed at day 6 p.i. When using a higher dose (MOI 0.1), infection could already be detected by immunofluorescence staining of N protein after 2 days indicating virus entry and replication (Figure 5A, Table 5). Confluent infection of around 60% of the cell layer was observed 12 days (MOI 0.001) or 8 days (MOI 0.1) after inoculation (Figure 5B). To test whether infectious virus was released from the MGN cells, the supernatant of infected MGN cells was taken after 10 days (Figure 5C, left panel) and inoculated to fresh MGN and Vero E6 reference cells (Figure 5C, right panel). The supernatant was able to again infect these cells indicating that MGN cells allow productive PUUV replication and formation of infectious virions (Figure 5C). To compare the replicative capacity of MGN cells with Vero E6, the standard cell line for hantaviruses, both cell lines were infected at two different MOI (0.1 and 0.001) and supernatant was harvested every two days. The resulting titrations showed a slightly delayed increase of the viral titer on MGN cells until day 8 when infected at MOI 0.1 if compared to the Vero E6 cells (Figure 5D). After day 8 titers reached the same level on both cell types. When using a lower infectious dose, the PUUV titer on MGN cells was about 2 log lower each day than on Vero E6 cells until day 14 when titers reached a plateau on the same level (Figure 5D). All other bank vole and common vole cell lines failed to replicate PUUV strain Vranica/Hällnäs and did not show any CPE at any time point (Figures 3A-3D, Table 5).

4. Discussion

This study revealed differences in the susceptibility of the tested bank vole and common vole cell lines that may reflect species-, evolutionary lineage- and tissue-specific differences in the susceptibility, replication capacity and CPE formation for various viruses (Table 5). The initial analysis of BVK168 cells confirmed results of a previous study for CPXV, VACV, SINV and USUV in terms of viral titers and CPE induction (38). Thus, neither the passage number of this cell line nor differences in the strain origin and passage history of the viruses used, influenced their interaction drastically.

Here, we developed novel cell lines that support productive replication of TULV and PUUV. We failed to detect any CPE of TULV in FMN cells and of PUUV in MGN cells, therefore these cell lines may reflect a natural infection in the reservoir that is believed to be non-cytolytic (12). Inoculation of TULV to kidney cell lines of both vole species resulted in TULV N-protein expression after incubation of up to 10 d.p.i. When testing cells for production of infectious particles in the supernatant, incubation after extensive washing had to be prolonged to 18 days for sufficient virus growth. Inoculation of the resulting supernatants from both cell lines was leading to complete infection of Vero E6 cells after 10 days, but only the FMN derived supernatant was able to re-infect this common vole cell line. This observation supports a host specificity of TULV for the common vole. In line, studies in wildlife small mammal populations detected TULV mainly in common vole, but only rarely in field vole and water vole, but not in the bank vole (7, 47). Experimental inoculation of bank voles with TULV could be shown, but only few animals seroconverted and antibody titers remained low (48). Therefore, FMN cell line might be suitable as a cell culture model system for TULV investigations. Future investigations should prove if the infection of FMN cells of the Central

evolutionary lineage of the common vole with an EST.S TULV strain reflect the situation in natural populations at a hybrid zone of the Central and Eastern evolutionary lineages of the common vole as described recently (49). Alternatively, the reference cell adaption of the TULV strain (50) might have influenced the host factor-dependence of the virus.

PUUV is another hantavirus that is commonly grown on Vero E6 cells. Here our novel bank vole cell line MGN of the Carpathian lineage was reliably infected and replicated PUUV strain Vranica/Hällnäs of the Northern Scandinavian (N-SCA) clade (51), but not the bank vole cell lines of the Western lineage (BVK168) and Eastern lineage (MGLU). The lacking replication of PUUV in the common vole cell lines might indicate the influence of host-specific factors. Unfortunately, the precise bank vole origin of the PUUV strain used is not known, but might be the Carpathian or Ural evolutionary lineage (52). Therefore, we are not able here to speculate about a lineage specificity of the bank vole cell line infection model, keeping in mind also the necessity of a genome-based analysis instead of the mtDNA-based lineage definition. Future studies should evaluate bank vole-derived PUUV isolates of different clades for their preference for cell lines of specific bank vole lineages.

The reservoir host of CPXV is currently a matter of debate. In our experiments the CPXV strain of cow origin replicated and induced CPE in all common and bank vole cell lines tested. VACV, another zoonotic orthopox virus, also efficiently replicated in the used bank vole and common vole cell lines. This was not surprising, as these viruses are able to replicate efficiently in a variety of different cell lines. The recent isolation of novel common vole-derived CPXV strains and the availability of common and bank vole animal models may allow a future evaluation of host specificity of CPXV (14, 53, 54).

Vector-transmitted pathogens must be highly adapted to both reservoir and vector. Interestingly, BVK168 cells have the capacity to efficiently replicate all tick- and mosquito-borne viruses investigated here. TBEV, RVFV and SINV replicated, at least to a certain level, in different other cell lines. The lacking CPE or low and late CPE formation observed here for TBEV in common and bank vole cell lines might reflect the situation in the natural host. In addition to the BVK168 cell line, all other cell lines supported replication of SINV to the same level as the control setup with Vero 76 cells. In line with these findings, SINV has been intensively studied for its growth characteristics in a broad range of vertebrate and also invertebrate cells with barely a cell line which did not support its propagation (55). Remarkably, the common vole brain cell line FMG did not support SINV replication. RVFV caused CPE in all four vole cell lines investigated and 3 out of 4 vole cells showed efficient titer production of the virus. Astonishingly, RVFV can infect a wide range of insect vectors, wild and domesticated animals as well as humans and replicates in a broad variety of cell lines (56). The virus was also shown to efficiently infect and replicate in airway epithelial cells of cotton rats (*Sigmodon hispidus*), another cricetid species (57). Based on these results, further investigations are urgently needed to prove whether vole species may represent a potential intermediate host of RVFV. A comparison of the replication in the four vole cell lines may indicate a kidney tissue preference for WNV and USUV – these viruses did not replicate at all in brain and lung cells used.

EMCV 1 replicated in three cell lines and induced a prominent CPE, except in FMG cells. This finding is in line with the broad host range of EMCV 1 including voles and squirrels. Infected rodents may play a role in virus spread when they occur in proximity to farms with infected swine (17). In addition, EMCV is a rapidly lytic virus that causes necrotic cell death.

Even SBV, a non-zoonotic pathogen with no evidence of a rodent-association (25), replicated to a certain level in all four tested cell lines. Non-zoonotic agents FMDV and MNV demonstrated a very low level of replication. FMDV was found to replicate in the BVK168 cell line, while MNV showed some level of replication in MGLU cells. Kidney derived cell lines of mice are able to propagate FMDV in culture (58) and the BHK-21[C13] (59) cell line is the cell line of choice for the industrial production of FMDV vaccines (60). The low or absent replication of MNV in the novel vole cell lines might be explained by the specific tropism of this virus for cells of the hematopoietic lineage such as macrophages and dendritic cells (61).

Our results show that MHV-68 was able to efficiently replicate in all four tested vole cell lines. This finding is unexpected as *DPOL* and *gB* genes of MHV-68 showed in a previous study a close phylogenetic similarity to those of wood mouse (*Apodemus sylvaticus*) and yellow necked mouse (*A. flavicollis*) associated rhadinoviruses, whereas a bank vole associated rhadinovirus formed a separate clade (62). Therefore, future studies in *Apodemus* derived cell lines and detailed *in vivo* studies are necessary to address this interesting finding.

5. Conclusion

Productive replication in the vole cell lines was observed for several viruses, for some without or delayed induction of CPE. Potent host cell lines for the propagation of PUUV and TULV were discovered that can be useful in revealing the yet unsolved virus-host interactions of these orthohantaviruses. These newly developed cell lines may represent a useful tool to study virus-cell interactions and to identify and characterize host cell factors involved in the replication of rodent associated viruses and thereby

mediating host adaptation. Furthermore, *in vitro* studies in these cell lines may allow first conclusions on the potential reservoir host(s) which has to be proven in targeted host animal experiments. Future investigations would profit from using virus strains that were not or only in low frequency passaged in non-reservoir cell lines to exclude potential artefacts generated by long-term passage of viruses.

Acknowledgements

The authors would like to thank Ulrike M. Rosenfeld for trapping of the bank vole, Hans-Joachim Pelz and Jens Jacob for providing the common voles, and Pawel Koteja for providing bank voles of the Carpathian lineage. We also would like to thank Sven Sander and Cornelia Steffen for excellent technical assistance, Indre Kucinskaite-Kodze and Aurelija Zvirbliene for providing mAb 5E11 and the lab of Bernd Köllner for providing the anti-TULV-N antibody TULV1. Thanks to Susanne Röhrs for providing animals of the bank vole colony of the Carpathian lineage, Martin Beer and Martin Groschup for continuous support and additional funding, and Isabella Eckerle for critical reading of the manuscript.

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Captions

Figure 1: Schematic representation of the workflow. Infection experiments of vole cell lines and back-titration on vole and reference cell lines (A) and positive control setup on the reference cell line for each virus (B). Pattern bars indicate identification code for titer diagrams in Figure 3. For reference cell lines see Table 1. Infection protocol for non-cytolytic hantaviruses differs from the protocol shown here and is described separately.

Figure 1:

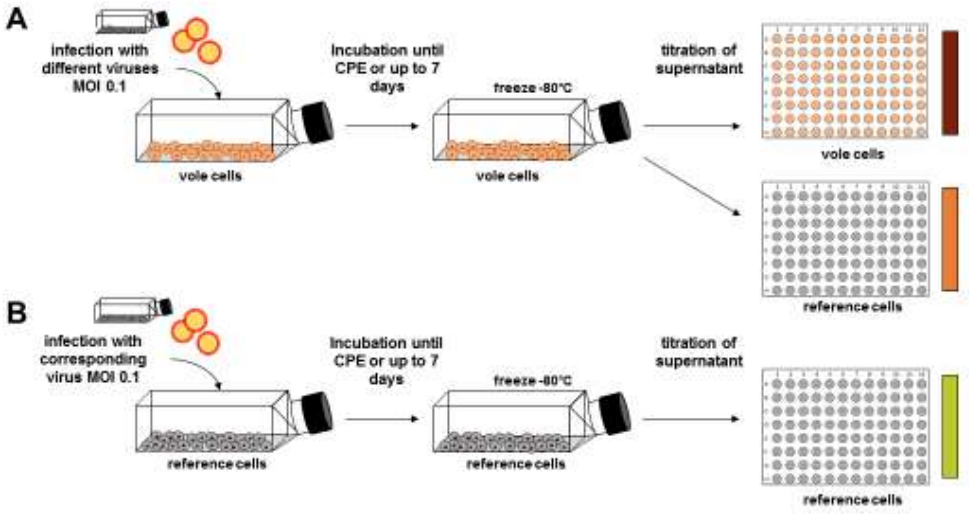

Figure 2: Cytopathic effect (CPE) on virus inoculated vole-derived cell lines BVK168 (A), MGLU (B), FMN (C), FMG (D). Phase contrast images are shown for time points with most prominent CPE in comparison to uninfected cells until day 7 post inoculation (p.i.). Abbreviations: viruses as they appear in Table 1: CPXV, Cowpox virus; EMCV, Encephalomyocarditis virus; FMDV, Foot and mouth disease virus; MHV-68, Murine gammaherpesvirus 68; MNV, Murine norovirus; RVFV, Rift Valley fever virus; SBV, Schmallenberg virus; SINV, Sindbis virus; TBEV, Tick-borne encephalitis virus; USUV, Usutu virus; VACV, Vaccinia virus; WNV, West Nile virus.

Figure 2:

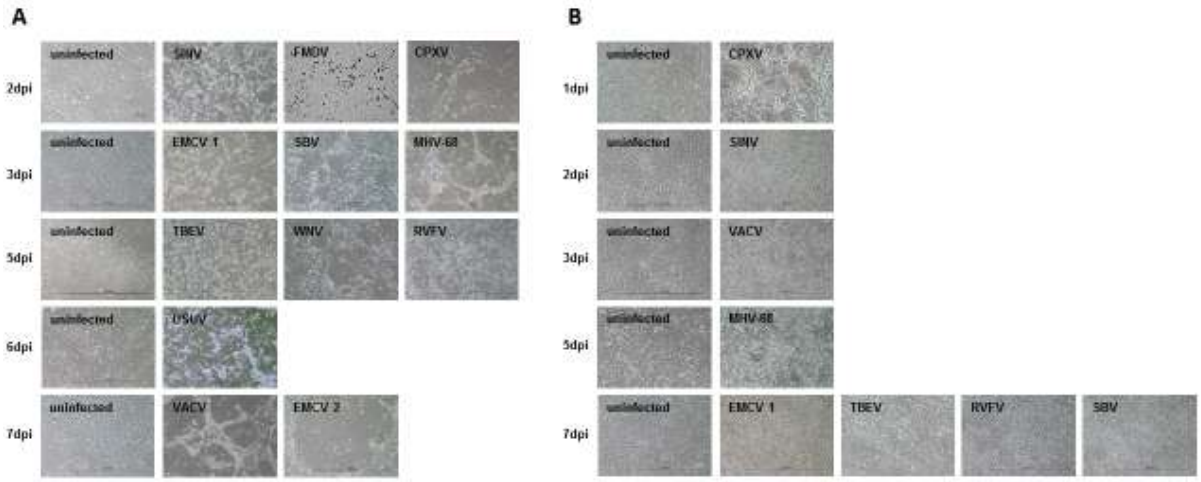

Figure 2
(continued):

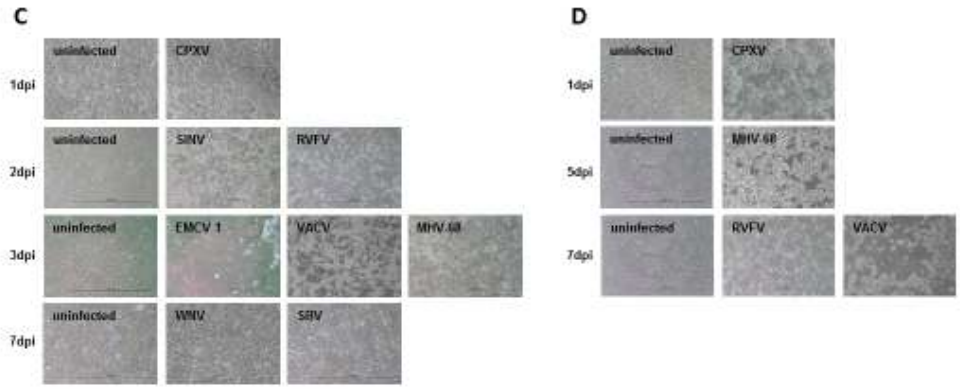

Figure 3: Results of virus titrations on vole cells in comparison to back-titration on reference cells. Vole cells were infected and titers observed from titration on vole cells are shown in brown bars. Titers from vole cell infection, titrated on reference cells

are shown in orange. Positive control infection was done on the reference cell line (Table 1) for each virus and titration is indicated by yellow bars. Titrations are shown as means with standard deviations of 4 replicates each. CPXV was titrated in two independent titrations and 8 replicates. Abbreviations: TCID, tissue culture infectious dose; CPXV, Cowpox virus; EMCV, Encephalomyocarditis virus; FMDV, Foot and mouth disease virus; MHV-68, Murine gammaherpesvirus 68; MNV, Murine norovirus; PUUV, Puumala orthohantavirus; RVFV, Rift Valley fever virus; SBV, Schmallenberg virus; SINV, Sindbis virus; TBEV, Tick-borne encephalitis virus; TULV, Tula orthohantavirus; USUV, Usutu virus; VACV, Vaccinia virus; WNV, West Nile virus.

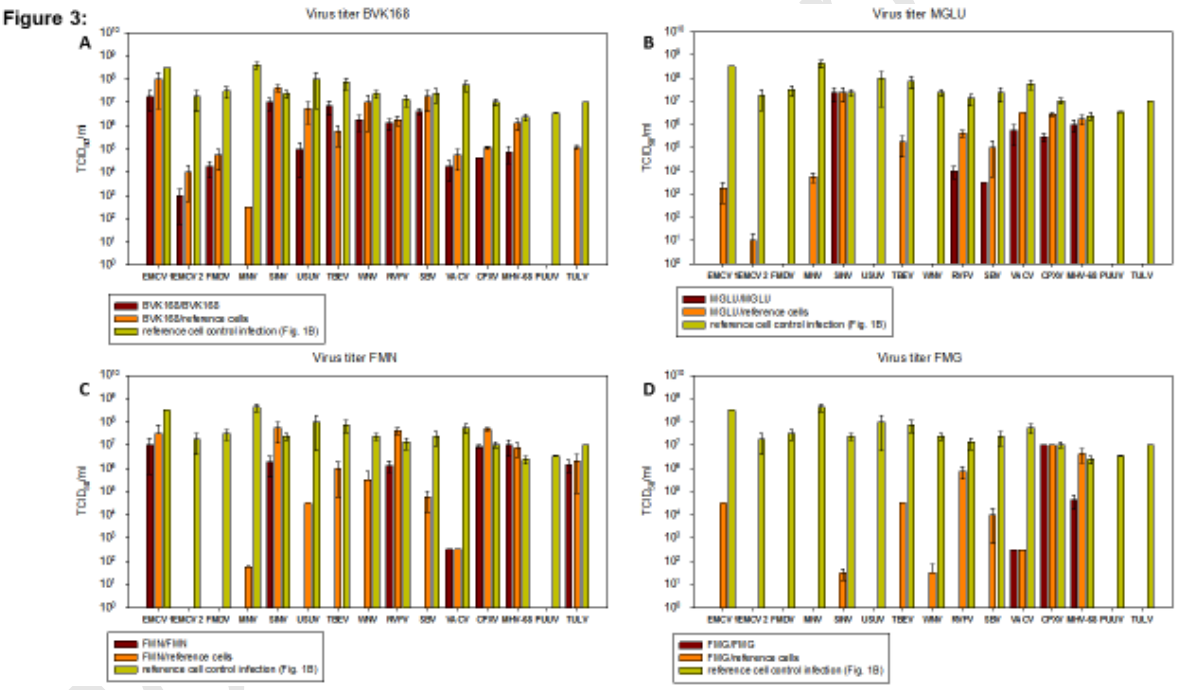

Figure 4: Reinfection of Vero E6 and bank vole and common vole-derived kidney cell lines with supernatants of vole cells inoculated with Tula orthohantavirus

(TULV). Infection of TULV on vole cell lines was monitored over 10 days by Western blot staining for TULV N protein (A and B) and β-Tubulin as loading control. Vero E6 and BVK168 or FMN cells were incubated with supernatant taken from TULV-infected BVK168 (C) or FMN (D) cells. Percentage of infected cells from C and D over time is shown in (E). Uninfected cells served as a control. Infected cells were detected by Western blot staining of hantaviral N protein.

Figure 4:

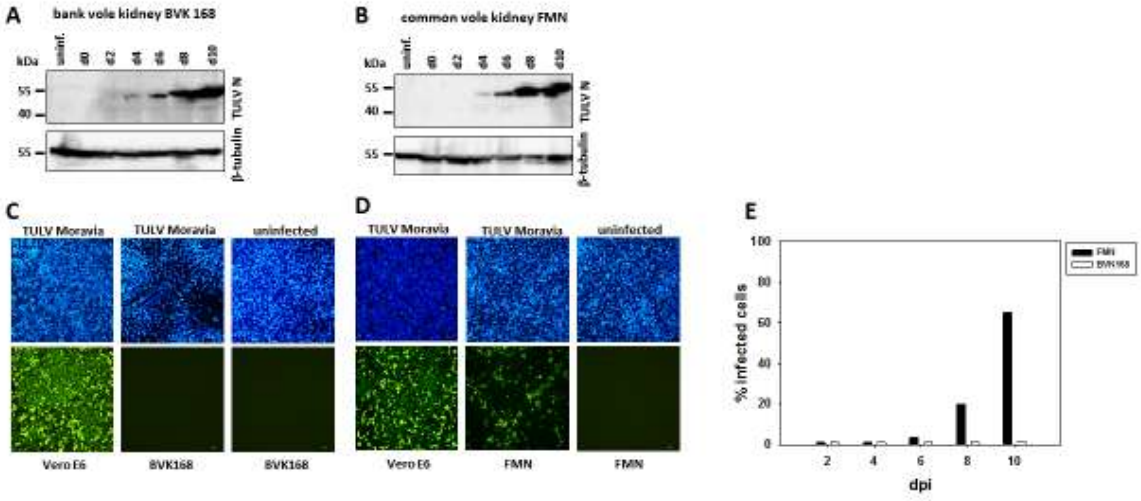

Figure 5: Immunofluorescence analysis of Puumala orthohantavirus (PUUV) infected MGN cells (A), percentage of infection (B), reinfection of MGN and Vero E6 cells (C), and PUUV titers on MGN cells (D). (A) MGN cells were inoculated with PUUV strain Vranica/Hällnäs at MOI of 0.1 or 0.001 for 2h, washed and incubated at 37°C for up to 14 days or left untreated. At the indicated time points, cells were fixed and stained with nucleocapsid (N)-protein specific monoclonal antibody 5E11 and an

Alexa fluor 488 labelled secondary anti-mouse antibody. Nuclei were stained with DAPI. Merge images are shown. (B) Percentage of infected cells from Fig. 5A. (C) Left panel: MGN and Vero E6 cells were incubated with PUUV strain Vranica/Hällnäs for 2h, washed and kept at 37°C for 10 days until supernatant was collected. Right panel: Vero E6 and MGN cells were incubated with supernatant of infected MGN cells. Infected cells were detected by immunostaining of hantaviral N protein. (D) PUUV titers on MGN cells in comparison to Vero E6 standard cell line at two different MOI. MGN (solid line) and Vero E6 (dashed line) cells were infected at MOI 0.1 and 0.001 as described. Titration of supernatants of PUUV-infected MGN cells was done on Vero E6 cells using indirect immunofluorescence test as described. Titers were calculated by the Spearman/Kärber method and mean titers of three experiments are given with standard deviation.

Figure 5:

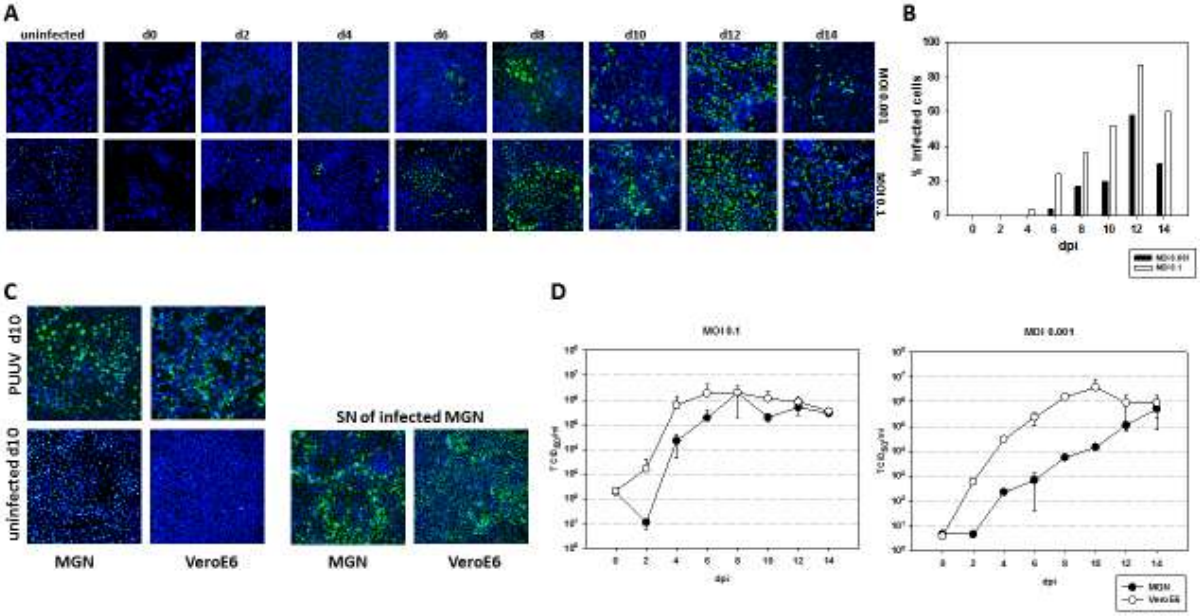

Table 1: Viruses tested for infectivity on established wild rodent cell lines.

Family	Genus	Group/Virus	Strain	Reference cell line	Zoonotic potential	Host/origin	Detection in rodents
Picornaviridae	*Cardiovirus*	Encephalomyocarditis virus 1 (EMCV 1)	Hungary IV29	BHK-21	+	Rodents[17]	yes
Picornaviridae	*Cardiovirus*	Encephalomyocarditis virus 2 (EMCV 2)	RD 1338 (D28/05)	BHK-21	+	Rodents[17]	yes
Picornaviridae	*Aphthovirus*	Foot and mouth disease virus (FMDV)	A24 Cruzeiro	BHK-21	-	cattle[63]	no
Caliciviridae	*Norovirus*	Murine norovirus (MNV)	S99	RAW 264.7	-	Rodents[61]	yes
Togaviridae	*Alphavirus*	Sindbis virus (SINV)	Hooded Crow (JX570540)	Vero 76	+	Birds[21]	yes
Flaviviridae	*Flavivirus*	Usutu virus (USUV)	USUV Europe 3 (BH65/11-02-03)	Vero 76	+	Birds[20]	yes
Flaviviridae	*Flavivirus*	Tick-borne encephalitis virus (TBEV)	TBEV Neudoerfl	Vero B4	+	Rodents[64]	yes
Flaviviridae	*Flavivirus*	West Nile virus (WNV)	WNV Austria (lineage 2)	Vero B4	+	Birds[65]	yes
Phenuiviridae	*Phlebovirus*	Rift Valley fever virus (RVFV)	MP-12	Vero 76	+	Ruminants[56]	yes
Hantaviridae	*Orthohantavirus*	Puumala orthohantavirus (PUUV)	Vranica/Hällnäs	Vero E6	+	Rodents[35]	yes
Hantaviridae	*Orthohantavirus*	Tula orthohantavirus (TULV)	Moravia	Vero E6	(+)	Rodents[35]	yes
Peribunyaviridae	*Orthobunyavirus*	Schmallenberg virus (SBV)	BH80/11	BHK-21	-	Cattle[69]	no
Poxviridae	*Orthopox virus*	Vaccinia virus (VACV)	Ankara 091	Vero E6	+	Rodents[66]	yes
Poxviridae	*Orthopox virus*	Cowpox virus (CPXV)	2	Vero 76	+	Cattle[67]	yes
Herpesviridae	*Rhadinovirus*	Murid herpesvirus 4	Murine gammaherpesvirus 68	BHK-21	-	Mice[68]	yes

Table 2: Cytopathic effect (CPE) of viruses on reference cell lines.

days p.i.	EMCV 1 BHK-21	EMCV 2 BHK-21	FMDV BHK-21	MNV RAW 264.7	SINV Vero 76	USUV Vero 76	TBEV Vero B4	WNV Vero B4	RVFV Vero 76	SBV BHK-21	VACV Vero E6	CPXV Vero 76	MHV-68 BHK-21
1	++++, f	-	++++, f	+	+	-	-	-	-	+	+	++++, f	-
2		(+)		++++, f	++++, f	+	(+)	-	+	++	++		++
3		++				++	+	(+)	+++, f	++++, f	++++, f		++++, f
4		++++, f				++++, f	+	+					
5							++	++					
6							+++, f	+++, f					
7													

Abbreviations: p.i., post inoculation; EMCV, Encephalomyocarditis virus; FMDV, Foot and mouth disease virus; MNV, Murine norovirus; SINV, Sindbis virus; USUV, Usutu virus; TBEV, Tick-borne encephalitis virus; WNV, West Nile virus; RVFV, Rift Valley fever virus; SBV, Schmallenberg virus; VACV, Vaccinia virus; CPXV, Cowpox virus; MHV-68, Murine gammaherpesvirus 68; -, no CPE; (+), <20% visible CPE; +, 20-40% visible CPE; ++, 40-60% visible CPE; +++, 60-80% visible CPE; ++++, >80% visible CPE; f, culture frozen until further use. Viral cytopathic effect in cell lines was evaluated semi-quantitative.

Table 3: Cytopathic effect (CPE) of viruses in bank vole derived kidney cell line BVK168 (A) and lung cell line MGLU-2-R (B).

A

bank vole kidney BVK168													
	days p.i.	EMCV 1	EMCV 2	FMDV	MNV	SINV	USUV	TBEV	WNV	RVFV	SBV	VACV	CPXV	MHV-68
	1	(+)	-	-	-	-	-	-	-	-	-	(+)	+	+
	2	+	-	-	-	++++, f	-	(+)	-	+	+	+	+++, f	++
	3	++++, f	-	+++,f	-		-	+	(+)	++	+++, f	+		++++, f
	4		-		-		+	++	++	++		+		
	5		-		-		++	+++, f	+++, f	+++, f		+		
	6		+		-		+++, f					++		
	7		++, f		-, f							+++, f		

B

bank vole lung MGLU-2-R

days p.i.	EMCV 1	EMCV 2	FMDV	MNV	SINV	USUV	TBEV	WNV	RVFV	SBV	VACV	CPXV	MHV-68
1	-	-	-	-	++	-	-	-	-	-	-	++++, f	-
2	-	-	-	-	++++, f	-	-	-	-	-	+		-
3	(+)	-	-	-		-	-	-	-	-	+++, f		++
4	(+)	-	-	-		-	-	-	+	-			++
5	+	-	-	-		-	-	-	+	-			++++, f
6	++	-	-	-		-	(+)	-	+	+			
7	++, f	-, f	-, f	-, f		-, f	(+), f	-, f	++, f	+, f			

Abbreviations: p.i., post inoculation; EMCV, Encephalomyocarditis virus; FMDV, Foot and mouth disease virus; MNV, Murine norovirus; SINV, Sindbis virus; USUV, Usutu virus; TBEV, Tick-borne encephalitis virus; WNV, West Nile virus; RVFV, Rift Valley fever virus; SBV, Schmallenberg virus; VACV, Vaccinia virus; CPXV, Cowpox virus; MHV-68, Murine gammaherpesvirus 68; -, no CPE; (+), <20% visible CPE; +, 20-40% visible CPE; ++, 40-60% visible CPE; +++, 60-80% visible CPE; ++++, >80% visible CPE; f, culture frozen until further use. Viral cytopathic effect in BVK168 cells was evaluated semi-quantitative.

Table 4: Cytopathic effect (CPE) of viruses in common vole derived kidney cell line FMN-R (A) and brain cell line FMG-R (B).

A

B

common vole brain

common vole kidney

FMG-R

FMN-R

days p.i.	EMCV 1	EMCV 2	FMDV	MNV	SINV	USUV	TBEV	WNV	RVFV	SBV	VACV	CPXV	MHV-68
~~days p.i.~~	~~EMCV 1~~	~~EMCV 2~~	~~FMDV~~	~~MNV~~	~~SINV~~	~~USUV~~	~~TBEV~~	~~WNV~~	~~RVFV~~	~~SBV~~	~~VACV~~	~~CPXV~~	~~MHV-68~~
1	-	-	-	-	-	-	-	-	-	-	-	++++, f	-
1	(+)	-	-	-	+	-	-	-	++	-	(+)	++++, f	-
2	-	-	-	-	-	-	-	-	-	-	(+)		-
2	++	-	-	-	++++, f	-	-	-	+++, f	-	+		+
3	-	-	-	-	-	-	-	-	+	-	+		(+)
3	++++, f	-	-	-	-	-	-	-		-	+++, f		++++, f
4	-	-	-	-	-	-	-	-	++	-	+		++
4	-	-	-	-	-	-	-	(+)	++	-	++		+++, f
5	-	-	-	-	-	-	-	-		-			
5	-	-	-	-	-	-	-	+	++	-	++		
6	-	-	-	-	-	-	-	-	++	-	++		
6	-	-	-	-	-	-	-	++		-			
7	-, f	-, f	-, f	-, f	-, f	-, f	-, f	-, f	+++, f	-, f	+++, f		
7		-, f	-, f	-, f		-, f	-, f	++, f		(+), f			

Abbreviations: p.i., post inoculation; EMCV, Encephalomyocarditis virus; FMDV, Foot and mouth disease virus; MNV, Murine norovirus; SINV, Sindbis virus; USUV, Usutu virus; TBEV, Tick-borne encephalitis virus; WNV, West Nile virus; RVFV, Rift Valley fever virus; SBV, Schmallenberg virus; VACV, Vaccinia virus; CPXV, Cowpox virus; MHV-68, Murine gammaherpesvirus 68; -, no CPE; (+), <20% visible CPE; +, 20-40% visible CPE; ++, 40-60% visible CPE; +++, 60-80% visible CPE; ++++, >80% visible CPE; f, culture frozen until further use. Viral cytopathic effect in BVK168 cells was evaluated semi-quantitative.

Table 5: Summary of cell lines and viruses tested.

Virus	Common vole								Bank vole											
	FMN				FMG				BVK168				MGLU				MGN			
	CPE	susceptibility	titration ref. cells	titration vole cells	CPE	susceptibility	titration ref. cells	titration vole cells	CPE	susceptibility	titration ref. cells	titration vole cells	CPE	susceptibility	titration ref. cells	titration vole cells	CPE	susceptibility	titration ref. cells	titration vole cells
zoonotic, rodent																				
PUUV	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	++	++
TULV	-	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	n.d.			
CPXV	+	+	++	++	+	+	++	++	+	+	+	+	+	+	++	++	n.d.			
VACV	+	+	+	+	+	+	+	+	+	+	+	+	+	+	++	++	n.d.			
EMCV 1	+	+	+	++	-	+	+	-	+	+	++	++	+	+	+	-	n.d.			
EMCV 2	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	n.d.			
zoonotic, rodent, with vector																				
TBEV	-	+	++	-	-	+	+	-	+	+	++	++	(+)	+	++	-	n.d.			
non-zoonotic, rodent																				
MNV	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	n.d.			
MHV-68	+	+	++	++	+	+	++	++	+	+	++	++	+	+	++	++	n.d.			
zoonotic, other, with vector																				
USUV	-	+	+		-	-	-	-	+	+	+	++	-	-	-	-	n.d.			
WNV	+	+	++	-	-	-	-	-	+	+	++	++	-	-	-	-	n.d.			
RVFV	+	+	++	++	+	+	++	-	+	+	++	+	+	+	++	++	n.d.			
SINV	+	+	++	++	-	-	-	-	+	+	++	++	+	+	++	++	n.d.			
non-zoonotic, other																				
FMDV	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	n.d.			
non-zoonotic, other, with vector																				
SBV	(+)	+	+	-	-	+	+	-	+	+	++	++	(+)	+	+	++	n.d.			

Abbreviations: ref., reference; CPE, cytopathogenic effect; -, none; (+), low/late CPE; +, moderate replication (30-70% of control infection); ++ high replication (>70% of control infection); n.d., not determined; PUUV, Puumala orthohantavirus; TULV, Tula orthohantavirus; CPXV, Cowpox virus; VACV, Vaccinia virus; EMCV, Encephalomyocarditis virus; TBEV, Tick-borne encephalitis virus; MNV, Murine norovirus; MHV-68, Murine gammaherpesvirus 68; USUV, Usutu virus; WNV, West Nile virus; RVFV, Rift Valley fever virus; SINV, Sindbis virus; FMDV, Foot and mouth disease virus; SBV, Schmallenberg virus.