Limited environmental stability of infectious porcine endogenous retrovirus type C; Usage of reverse transcriptase in combination with viral RNA as markers for infectious virus

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Abstract
Introduction: Porcine endogenous retroviruses (PERVs) are an integral part of the pig genome with infectious potential, as shown in vitro.

Hypothesis/gap statement: In view of nonclinical and clinical xenotransplantation, data are essential that give an insight into viral pathogenicity. This includes PERV's environmental stability and environmental risk.

Aim: We analyzed two ecotropic PERV-C (PERV-C[1312] and -[5683]), monitoring cell-free culture supernatants of infected ST-IOWA cells at various time intervals at room temperature (22°C +/− 1°C). The virus was stored in the presence or absence of sterile wood litter, as used for large animal husbandry. This approach was set to determine the environmental stability of exogenous PERV-C at defined conditions for the first time.

Methodology: Reverse transcriptase (RT) activity and viral RNA were monitored for up to 57 days and remaining infectivity of supernatant without wood litter was tested from day 7 onwards on naïve ST-IOWA cells.

Results: Results show that viral RNA decreases but remains detectable over the whole observation period, whereas RT activity showed 83%–96% reduction from day 7 on. This effect was stronger in the presence of wood litter and fresh harvested virus was more stable than frozen virus stocks. Even under these optimal conditions, no infectivity was shown for viral RNA-positive and RT-reduced supernatant harvested at day 7.

Conclusion: The results confirm that PERV-C is less stable and the reduction of RT activity is accompanied by reduced infectivity, independently of existing viral RNA. The combination of both RT and viral RNA measurement is a suitable method to differentiate infectious PERV-C.

Keywords
environmental risk, environmental stability, PERV-C, porcine endogenous retrovirus (PERV), viral pathogenicity, xenotransplantation (XTx)
1 | INTRODUCTION

Endogenous retroviruses (ERV) and related sequences are integral parts of the eukaryotic genome that evolved by retroviral infection of germlines millions of years ago. A great proportion of beta-type ERV, including some classes of human endogenous retroviruses, are no longer replication competent. Some retroviruses belonging to the gamma-retrovirus group have preserved their infectious capacities, such as murine leukemia virus (MuLV), koala retrovirus, and feline leukemia virus (FeLV). Here, we focus on porcine endogenous retrovirus (PERV), which is also a gamma-retrovirus and an integral part of the pig genome. It comprises three major classes, PERV-A, -B, and -C, present in the genome as proviruses. Most of them are truncated and impaired and not all pigs harbor PERV-C. Nonetheless, a number of replication-competent full length PERV-A, -B, and -C viruses from different pig breeds and cell lines have been isolated and were tested in vitro. Even if there is no documented in vivo infection or zoonosis, the relevance of PERV as an infectious agent is taken into account. The absence of evidence remains a hurdle for xenotransplantation (XTx) since the development of pig-derived cell-based therapeutics inevitably requires maintenance of virus safety. Besides characterization of virus load and expression in the animal donor, virus environmental stability and putative transmissibility are important aspects at least from an academic point of view. The capacity of PERV to maintain infectivity outside the host is not described. For other retroviruses such as FeLV, it has been shown that cats that were infected with FeLV release retroviruses in feces stable without refrigeration for up to 42 days post collection. Naïve cats regularly exposed to FeLV-positive feces seroconverted, proving that infection took place. Similar results have been observed for mice and MuLV. Regarding replication-competent viruses and/or vectors used for gene therapy, environmental risk assessment (ERA) is an important part of preventing unwanted release. For XTx, particularly in view of genetically modified (transgenic or knock-out) pigs, ERA is required to assess a hypothetical virus transmission in housing as well as to fulfill biosafety requirements prior to nonclinical and clinical studies.

Here, the environmental stability of PERV-C was analyzed while storing virus particles in tissue culture medium at controlled laboratory room temperature, with and without sterilized wood litter used for large animal husbandry. To set a basis for further experiments, we addressed the question of how long viral infectivity persists under these defined conditions.

The scenario chosen differs from natural barn conditions, which are much more complex in terms of moisture, oxygen content, different layers and temperatures, salts, urea, and carbon compounds. Here, the presence of shed pig cells or cell conglomerates may protect intercellular, budded virus from degradation and may prolong virus stability. However, in vitro infection assays using PERV-C virus isolated from barns are practically challenging due to contamination and virus quantity. If anything, PERV-C virus may not be excreted in sufficiently detectable amounts and in the pig it is present on the RNA level, possibly on the protein level only. Furthermore, it has not been isolated from whole organs, tissues (primary cells), or fluids including blood (cells), with the exception of Phytohemagglutinin (PHA)-activated Peripheral Blood Mononuclear Cells (PBMCs) and porcine aorta endothelial cells from Yucatan and Gottingen minipigs.

PERV-C is not present in all pigs but is relevant to XTx since it may recombine with PERV-A to PERV-A/C; thus, the infectious potential of PERV-C is important to analyze. In vivo pig infection studies using recombinant PERV-C are extremely difficult to perform. No data on environmental stability have thus far been published. For this reason, and in view of ERA, we tested molecularly cloned, functional, and infectious PERV-C (PERV-C[1312], AM229312.2,19 and PERV-C[5683], KY352351.1) containing cell culture supernatants (SNs) from ST-IOWA producer cells that have been incubated for up to 57 days (8 weeks) at ambient temperature in the presence or absence of sterile wood litter. Reverse transcriptase (RT) activity and presence of viral RNA (vRNA) was monitored over the whole period. The remaining infectivity of PERV-C present in SN was analyzed at day 7 by inoculation of naïve ST-IOWA cells with these SNs. At that time, samples clearly showed strongly reduced RT activity while vRNA copy number was still present. RT activity and PERV-C env vRNA of these inoculated cells was monitored for 7 weeks and compared with ST-IOWA cells treated with the initial SN used for tests of stability.

2 | METHODS

2.1 | Cell lines and viruses

PERV-C was harvested from the producer cell line ST-IOWA carrying either molecular clone PERV-C(1312), AM229312.2,19 or PERV-C(5683), KY352351.1. PERV susceptible, RT-negative ST-IOWA cells were used as controls. ST-IOWA cells are a well described and established cell line for infection assays as they are PERV-C negative, susceptible to PERV-C infection, and free of functional PERV-A or -B, given by the absence of RT activity besides a basal background of RNA copies independent of infection. This RNA background is commonly known and has been described before. The culture of cells was performed as described earlier.

2.2 | Virus stability testing, experimental setup

For virus stability test, 10 T25 cell culture flasks (Greiner, Germany) were prepared with either 20 ml virus containing cell culture SN or 40 ml virus containing cell culture SN supplemented with 1.5 g autoclaved wood litter commonly used for pig barns as bedding material (b) (Allspan German Horse, Germany) and stored at controlled room temperature (22°C +/− 1°C). The ratio of wood litter to cell culture SN was slightly above saturation to avoid the sample drying out. The SN tested was, on the one hand, freshly harvested, both from PERV-C(1312) and PERV-C(5683) producing ST-IOWA cells and on the other hand previously harvested from ST-IOWA producer cells and stored at −80°C until use. The initial RT activity was determined between 34 and 217
RESULTS

2.3 | in vitro infection of ST-IOWA cells

For in vitro infection, triplicates of $2 \times 10^5$ ST-IOWA cells/well, seeded in six-well cell culture plates (Sarstedt, Germany) 24 h prior to infection, were incubated with 1 ml/well virus containing SN from b(−) samples of d7 (day 0, input virus for infection) and incubation maintained for 24 h. After washing the cells three times with 2 ml/well phosphate buffered saline to remove input virus, cells were further cultivated for 49 days with passaging of cells every 3–4 days in equal split ratios. The storage of cell-free SN containing PERV-C(1312) and PERV-C(5683) that were not stored at −80°C before testing was more stable than virus from −80°C animal bedding of either fresh (SN, b[−]) or frozen (SN, −80°C, b[+]) origin, stored at ambient temperatures and monitored for 57 days. Ctr(−): SN of ST-IOWA cells with (ctr[−], b[+]) or without (ctr[−], b[−]) animal bedding

2.4 | Quantification of PERV

Detection and quantification of PERV-C env vRNA was performed as described using primers Env-C for 2 and Env-C-R3_AWG for amplification. One-step Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) was performed in a LightCycler® 480 (Roche Life Science, Germany) using the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Germany) as recommended by the manufacturer. The storage of cell-free SN containing PERV-C(1312) and PERV-C(5683) from ST-IOWA producer cells at controlled room temperature (22°C +/- 1°C) with [b(+] and without [b(−)] sterilized wood litter, as used for large animal husbandry, led to a continuous reduction of viral enzymatic RT activity (Figure 1, Table 1). The initial RT activity was determined with 217 mU/ml for PERV-C(1312) and 34 mU/ml for PERV-C(5683) in SN that was freshly harvested and 193 mU/ml for PERV-C(1312) and 56 mU/ml for PERV-C(5683) in SN that was collected before and stored at −80°C until testing. Virus-containing SN showed approximately 33% mean reduction within 1 day when stored in b(−) compared to 66% when stored in b(+) flasks (Table 1). At d7, only weak RT activity (15–0 mU/ml +/- 1.2 mU/ml mean standard deviation (SD)) was detectable in b(+) samples. In b(−) samples, RT activity remained between 6 and 43 mU/ml +/- 1.4 mU/ml mean SD. Until d29, most (6 out of 8) RT activities were below 6 mU/ml and at d57 there was only basal enzymatic activity left. Both the viruses tested (PERV-C[1312] and PERV-C[5683]) showed a similar pattern. Freshly harvested virus was more stable than virus from −80°C stocks when comparing the d1 reduction data (Table 1). Virus-free SN from PERV-C negative ST-IOWA cells, used as control, remained negative.

2.5 | RT activity assay

RT activity in cell-free SN of infected cells and producer cells was detected as described using primer pair C-Type RT Activity Kit (Cavidi, Sweden) according to the manufacturer’s instructions, protocol B. Samples were measured in triplicates and the mean value and standard deviation were determined.

3 | RESULTS

3.1 | Quantification of PERV-C RT activity

The storage of cell-free SN containing PERV-C(1312) and PERV-C(5683) from ST-IOWA producer cells at controlled room temperature (22°C +/- 1°C) with [b(+[)] and without [b(−)] sterilized wood litter, as used for large animal husbandry, led to a continuous reduction of viral enzymatic RT activity (Figure 1, Table 1). The initial RT activity was determined with 217 mU/ml for PERV-C(1312) and 34 mU/ml for PERV-C(5683) in SN that was freshly harvested and 193 mU/ml for PERV-C(1312) and 56 mU/ml for PERV-C(5683) in SN that was collected before and stored at −80°C until testing. Virus-containing SN showed approximately 33% mean reduction within 1 day when stored in b(−) compared to 66% when stored in b(+) flasks (Table 1). At d7, only weak RT activity (15–0 mU/ml +/- 1.2 mU/ml mean standard deviation (SD)) was detectable in b(+) samples. In b(−) samples, RT activity remained between 6 and 43 mU/ml +/- 1.4 mU/ml mean SD. Until d29, most (6 out of 8) RT activities were below 6 mU/ml and at d57 there was only basal enzymatic activity left. Both the viruses tested (PERV-C[1312] and PERV-C[5683]) showed a similar pattern. Freshly harvested virus was more stable than virus from −80°C stocks when comparing the d1 reduction data (Table 1). Virus-free SN from PERV-C negative ST-IOWA cells, used as control, remained negative.

3.2 | Quantification of PERV-C vRNA

PERV-C-containing SN [b(+)] and [b(−)] was quantified at days 0, 1, 2, 3, 7, 15, 29, 43, and 57 for presence of PERV-C env vRNA (Figure 2). According to the RT activity testing, vRNA in b(+) samples decreased faster and at higher percentage than in b(−) samples. Both virus strains tested showed comparable results at the end point (Table 1). Compared to RT activity, vRNA levels decreased much more gradually under b(−) conditions, and complete elimination was not attained after 57 days. Levels of 2 × 10^4 to 8 × 10^5 copies/µl PERV-C env vRNA remained, respectively. In general, vRNA sampled from freshly harvested PERV-C(5683) and PERV-C(1312) that were not stored at −80°C before testing was more stable and was less affected by storage at ambient temperature than the viral RT (Table 1).

3.3 | in vitro infection assay

Since mean RT activity was reduced by 96% in b(+) and by 83% in b(−) samples within 7 days of storage at ambient temperature, the remaining infectivity of PERV-C was analyzed. Here, naïve ST-IOWA...
cells were infected with SN from b(−) samples of PERV-C(1312) or PERV-C(5683) harvested at d7. None of the samples, either from fresh virus or the −80°C stock, which were stored for 7 days at room temperature, had the capacity to induce a productive PERV-C infection in ST-IOWA cells, despite the presence of vRNA (Figure 2). Virus inoculum from the initial fresh stock that remained frozen at −80°C for 7 days was used as positive control (Figures 3A and 3B, ctr[+], green bars) to confirm virus infectivity. Data show that ST-IOWA cells infected with ctr(+) inoculum became RT positive within 14 days post infection (Figure 3A). The results were confirmed by quantifying vRNA. Only cells infected with PERV-C(1312) or PERV-C(5683) as a positive control showed an increasing vRNA level that was clearly detectable at day 14 and remained stable until the end of the experiment at d49 (Figure 3B).

4 | DISCUSSION

Despite phylogenetic data on PERV, aspects of host pathogen interaction, and its relevance for XTx, little is known about the environmental stability and transmissibility of PERV-C. For PERVs, the possibility of an exogenous infection was exclusively addressed in vitro by host range and interference studies as well as various infection experiments in different species including nonhuman primates and first clinical trials in diabetic patients, showing no transmission of PERV. But even if this is more of an academic aspect, it is worth being investigated. Since all pigs carry at least PERV-A and -B, transmission between pigs has been excluded until now on the basis that viral superinfection does not occur and intact PERV particles have hardly been found in pigs even if de novo integration was shown. However, the environmental half-life of PERV has to be considered, for instance while planning experiments involving in vivo pig infections using functional and/or recombinant PERV-C and in view of clinical trials including patient monitoring even if it is a theoretical aspect. Regarding genetically modified pigs, ERA is required in order to assess potential virus transmission and to fulfill biosafety requirements. This is also important in view of founder pigs as well as donor pigs generated for XTx in animal breeding centers. To draw the complete picture for virus safety, knowledge of viral transmission routes including virus environmental stability is essential. Besides other guidance documents, the “Third WHO Global Consulta-
Infection of naïve ST-IOWA cells with porcine endogenous retrovirus type C (PERV-C) (1312) or PERV-C(5683) containing supernatant (SN) that was stored for 7d at ambient temperature without animal bedding of either fresh (SN, b−), d7, or frozen (SN, −80°C, b−), d7 origin. Virus inoculum from the initial fresh stock that remained frozen at −80°C for 7 days was used as positive control (SN, ctr+), d0, green bars) to confirm virus infectivity. Uninfected ST-IOWA cells (ctr−, b−) served as negative control. (A) Infection progression monitored by increasing reverse transcriptase (RT) activity. (B) Infection progression monitored by PERV-C env viral RNA (vRNA). As expected, ctr+ infected cells reveal a PERV-typical course of infection measured by RT activity and vRNA that increases in the beginning, reaching a peak at weeks 5–6 to flatten down at stabilized expression by about weeks 7–8.

Since PERV-C is not present in all pigs but may recombine with PERV-A to give the recombinant human-tropic and high-titer PERV-A/C,42,43 we investigated the environmental stability of two different PERV-C molecular virus clones. We stored PERV-C-containing cellular SNs at room temperature in the presence and absence of wood litter that was a major component of pig animal bedding over a period of 57 days. The results show that the RT activity is rapidly lost in the presence of wood litter within 7 days. This effect is possibly caused through an absorption of the fluid that may lead to a structural break of virus particles and irreversible decline of RT enzymatic activity. Furthermore, it is conceivable that wood litter contains inhibitors that have influence on the RT. Since a potential toxic effect of wood litter on cells cannot be excluded, we tested only SN from b− samples for later infection assay on ST-IOWA cells. Compared to RT activity, the vRNA remained detectable at high copy numbers during the whole period of 57 days.

In summary, the decrease in RT activity was accompanied by a loss of infectivity (Figures 1 and 3A), indicating that the viral RT is not only necessary but also a suitable surrogate marker for viral infectivity being used for further evaluation. The infectivity of PERV-C did not depend directly on the presence of vRNA since PERV-C env vRNA was still available at high amounts during the whole experiment. The results clearly show that RNA, utilized as gold standard for PERV detection, is not the only defining functional parameter for analyzing the infectious risk of PERV. Only intact viruses have the ability to infect host cells.
and to integrate their genomes. The probability of unwanted genomic integration of foreign vRNA, while possible, is unlikely, based on the infection results presented here. These data provides initial insights toward PERV environmental stability and infectivity outside its host.

5 | CONCLUSIONS

In this study, we analyzed the stability of two different PERV-C molecular clones, PERV-C(1312) and PERV-C(5683), in culture SN with and without sterile animal bedding material at controlled room temperature. Due to the fact that handling of genetically modified organisms or viruses requires an evaluation of potential environmental impact, we initiated this investigation to estimate how long PERV-C remains infectious at ambient temperature outside its host. This investigation is the first approach to determine the environmental stability of PERV-C and requires further analysis under conditions found in the actual barn husbandry setting. We showed in vitro by using a ST-IOWA cell based assay that even in cell culture medium at 100% humidity, the RT activity decreases continuously, resulting in a loss of infectivity within 7 days despite the presence of vRNA. The presence of vRNA without intact viral RT is not sufficient in itself to initiate a productive infection. For this reason, RT testing should be considered as an additional or surrogate marker to differentiate infectious PERV.

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AUTHOR CONTRIBUTIONS

Nicole Fischer performed the functionality studies, analyzed data, and drafted the manuscript. Barbara Gulich was involved in experimental setting and data analysis. Ralf R. Tönjes secured funding for the research, design of the project, and drafting of the manuscript. Antonia W. Godehardt partook in functional studies, design of the project, interpretation of data, and drafting of the manuscript.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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