Long-term absence of porcine endogenous retrovirus infection in chronically immunosuppressed patients after treatment with the porcine cell-based Academic Medical Center bioartificial liver


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<td>Di Nicuolo, Giuseppe; Cotugno Hospital, Lab of Virology D'Alessandro, Alba; Antonio Cardarelli Hospital, Center of Biotechnologies Andria, Barbara; Antonio Cardarelli Hospital, Center of Biotechnologies Scuderi, Vincenzo; Antonio Cardarelli Hospital, Hepatobiliary and Liver Transplant Unit Scognamiglio, Michele; Cotugno Hospital, Lab of Virology Tammaro, Angela; Antonio Cardarelli Hospital, Center of Biotechnologies Mancini, Antonio; Antonio Cardarelli Hospital, Center of Biotechnologies Cozzolino, Santolo; Antonio Cardarelli Hospital, Center of Biotechnologies Di Florio, Ernesto; Antonio Cardarelli Hospital, Dept of Anaesthesiology and Intensive Therapy, Liver Transplant Unit Bracco, Adele; Antonio Cardarelli Hospital, Center of Biotechnologies Calise, Fulvio; Antonio Cardarelli Hospital, Hepatobiliary and Liver Transplant Unit Chamuleau, Robert; Academic Medical Center, Department of Hepatology</td>
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Abstract

Background
Clinical use of porcine cell based bioartificial liver (BAL) support in acute liver failure as bridging therapy for liver transplantation exposes to the risk of transmission of porcine endogenous retroviruses (PERVs) to human. The risk may be enhanced when patients receive liver transplant and are subsequently immunosuppressed.

As further follow-up of previously reported patients (Di Nicuolo et al 2005), an assessment of PERV infection was made in the same patient population pharmacologically immunosuppressed for several years after BAL treatment and in healthcare workers (HCWs) involved in the clinical trial at that time.

Methods
Plasma and peripheral blood mononuclear cells (PBMCs) from eight patients treated with the Academic Medical Center- Bioartificial Liver (AMC-BAL), who survived to transplant, and thirteen HCWs, who were involved in the trial, were assessed to detect PERV infection. A novel quantitative real time polymerase chain reaction assay has been used.

Results
Eight patients who received a liver transplant after AMC-BAL treatment are still alive under long-term pharmacological immunosuppression. The current clinical follow-up ranges from 5.6 to 8.7 years after BAL treatment.

A new q-real-time PCR assay has been developed and validated to detect PERV infection. The limit of quantification of PERV DNA was \( \geq 5 \) copies per \( 1 \times 10^5 \) PBMCs. The linear dynamic range was from \( 5 \times 10^0 \) to \( 5 \times 10^6 \) copies. In both patients and HCWs, neither PERV DNA in PBMCs nor PERV RNA in plasma and PBMC samples have been found.

Conclusions
Up to 8.7 years after exposure to treatment with porcine liver cell based BAL, no PERV infection has been found in long-term immunosuppressed patients and in HCWs by a new highly sensitive and specific q-real-time PCR assay.
Key words: bioartificial liver-hepatocytes-PERV-pig-xenotransplantation

Abbreviations:

ALF: acute liver failure; AFLP: acute fatty liver of pregnancy; AMC: Academic Medical Center;
BAL: bioartificial liver; HAV: Hepatitis A Virus; HBV: Hepatitis B Virus; HCW: Healthcare
Workers; HEK-293: human embryonic kidney cells; MOF: multi-organ failure; MSP: mushroom
poisoning; OLT: orthotopic liver transplantation; PBMCs: peripheral blood mononuclear cells;
PBS: phosphate buffered saline; PERV: porcine endogenous retrovirus; PK-15: porcine kidney
epithelial cell line; q-real-time PCR: quantitative polymerase chain reaction; SCID: severe
combined immune deficient; WD: Wilson Disease.
Introduction

Acute liver failure (ALF) is a rare, but devastating syndrome in which people with no previous history of liver disease can develop fatal liver disease within weeks of the first symptoms. In the USA and Europe each year more than 7,500 patients develop ALF. The majority of these patients die. ALF is characterised by massive liver cell death leading to loss of essential metabolic and synthetic functions and a deranged inflammatory response. Consequently, jaundice, bleeding, hepatic coma and multi-organ failure (MOF) develop rapidly.

The cause of ALF includes, among others, acute viral infections, such as hepatitis A, B and C, poisoning, and rare inherited metabolic diseases (1).

ALF has a high mortality rate: 50-90% (1) and in most cases liver transplantation (OLT) is the only treatment with proven efficacy. OLT is, however, not always possible because of the shortage of available organs for transplantation. However, there is a significant proportion of patients (20-40%) whose liver can recover if sufficient bridging time is provided.

For these reasons, a variety of extracorporeal hepatic assist devices has been developed to provide detoxification and/or metabolic support in patients with ALF (2).

The use of porcine liver cell-based extracorporeal support exposes the patients to the risk of xenogenic infectious agents. The major concern is the possibility of cross species transmission of porcine endogenous retroviruses (PERVs).

Pigs harbour many PERV sequences in their genome (3-8). It has been shown that PERVs are able to infect selected human cells in vitro (9-13). Extra-corporeal plasma perfusion through BAL may imply an hypothetic transfer of PERV infective particles from porcine liver cells in bioreactors to patient’s plasma during treatment. The risk may be enhanced when patients receive liver transplant and are long-term pharmacologically immunosuppressed. Although no PERV infections have been found in non-human primates exposed to porcine cells or organs (14-19), neither in human recipients parenteral exposed to porcine organs nor cells in xenotransplantation clinical trials (20-28), the most important regulatory agencies worldwide have designed very strict guidelines on
research in xenotransplantation in human subjects, to safeguard their health and the general population (29,30).

Between 2000 and 2003 fourteen patients with ALF waiting for OLT were treated with a porcine liver extracorporeal support system, named Academic Medical Center Bioartificial Liver (AMC BAL), in a phase I clinical trial (31). The AMC-BAL is a bioreactor charged with freshly isolated porcine liver cells connected outside the body to the plasma circulation of the patient. In contrast to other BAL systems screened for PERV transfer, in the AMC-BAL there is direct contact between patient’s plasma and the porcine liver cells without membrane interposition and liver cells are directly oxygenated by local oxygen capillaries (32). This internal organization allows to realize a bi-directional mass transfer, but, however, could be permissive for PERV transfer. During clinical application patient’s plasma is separated by a plasmapheresis apparatus, with a plasma filter of 0.47 µm pore size, and perfused through the bioreactor charged with 10 billions of viable porcine hepatocytes, as described elsewhere (32) (Fig.1-2).

Fourteen patients aged 20 to 56 years were included in the study. The causes of ALF were hepatitis B infection in seven, hepatitis A infection in one, acute fatty liver of pregnancy (AFLP) in one, Wilson disease in one, injury by mushroom intoxication in one and no definite etiology in three patients. The patients received one to two BAL treatments for a total time ranging from 4 to 35 hours: no adverse effects related to BAL treatment were reported. Thirteen patients received OLT: eight of them are now living under pharmacological immunosuppression regimen, four patients died because of OLT complications, one patient died two years after BAL treatment because of a second organ rejection. One patient did not receive OLT as spontaneous liver regeneration occurred.

This phase I clinical study in 14 ALF patients showed improvements in hemodynamics and diuresis, in the neurological state and several biochemical parameters and proved the safety of the treatment concerning the risk of transmission of PERV infection to patients. We found that patient’s plasma samples collected at the end of BAL treatment were positive for PERV DNA, most probably due to porcine liver cell lysis. Furthermore, the PERV DNA was cleared within two weeks post-
treatment and no PERV RNA was detected in plasma and PBMCs from patients treated with the AMC-BAL, with a follow-up of 2 years (33).

In the current study we evaluated the BAL treated patients who were chronically immunosuppressed after a post-treatment follow-up from 5.6 to 8.7 years. We used a new developed quantitative real-time PCR assay (q-real-time PCR) more sensitive than the previous one (33). In addition we evaluated PERV infection in healthcare workers (HCWs) who were involved in preclinical and clinical phases of trial. In no patient and none of the HCWs PERV infection had occurred.

This is the first report which supports the absence of PERV infection in transplant survivors who were long-term immunosuppressed after exposure to a porcine liver cell based bioartificial liver.
Materials and Methods

Study population

Eight living patients treated with the AMC-BAL between 2000 and 2003 were included in the study. All were chronically immunosuppressed because of receiving a liver transplant from a few hours to a few days after BAL treatment. One living patient, who recovered after BAL treatment without the need for a graft, has not agreed to the study. Frozen PBMCs and plasma samples of one patient, who died two years after BAL treatment because of a second organ rejection, have been included in the study to exclude PERV infection at that time. As comparison to previously reported data (33), frozen plasma and PBMC samples, collected from each patient immediately after BAL treatment and at the first time point of follow-up, were also assessed in this study.

In addition thirteen HCWs frequently exposed to pig tissues or blood containing PERV genomes or to in vitro cultures of porcine kidney epithelial cells (PK-15) and human embryonic kidney cells (HEK-293) infected with PERV, were included in the study. This group includes two surgeons, two anesthesiologists and three nurses, four biologists, two virologists.

All patients and HCWs enrolled in the study signed a written informed consent.

Clinical specimens

Whole EDTA blood samples from patients and HCWs were centrifuged at 2,500 rpm for 5 min and plasma was stored at -80°C.

PBMCs were isolated from nine ml of whole blood by gradient centrifugation method using Lymphoprep™ (Axis-Shield, Oslo Norway), according to manufacturer’s instructions. Cell pellets, corresponding to 1x10^5 PBMCs, were prepared and stored at -80 °C. PBMCs from three voluntary healthy donors were collected as source of human DNA control. PERV-producing PK15 and HEK 293 cells, infected with PK15 supernatant as previously reported (33), were used as PERV-positive controls. Uninfected HEK 293 cells were used as PERV-negative control.

Construction of PERV containing plasmid (p-PCR-PERV)
PERV RNA was isolated from 200 µl of cell culture supernatant of PK15 cells by using the High Pure Viral RNA Kit (Roche Diagnostics, Mannheim, Germany). Reverse transcription was performed with random hexamer primers by using Transcriptor High Fidelity cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany). DNA templates were amplified in a 151 bp amplicon of a highly conserved region of PERV polymerase (pol) gene, produced with primers PERV 1-F and PERV 1-R (Table 1). Amplified DNA products were purified and cloned using the TA Cloning® kit (Invitrogen Corporation) resulting in plasmid PCR-PERV (p-PCR-PERV). Confirmation of p-PCR-PERV insert was done by sequencing. The plasmid copy number was determined by measuring the optical density at 260 nm of plasmid DNA. An aliquot of 1x10^8 copies of PERV plasmid DNA was freeze-dried and stored at 4°C.

*Nucleic Acids purification*

DNA was extracted from 1x10^5 human PBMC pellets diluted in 200 µl of PBS or from 200 µl of plasma by using HighPure™ Template Preparation kit (Roche Diagnostics, Mannheim, Germany), according to manufacturer’s instructions. Aliquots of 1x10^5 PERV infected cells (PK15 and HEK 293 cells *in vitro* infected) and HEK 293 uninfected cells were included in each batch of DNA extraction as positive and negative controls, respectively. Isolated DNA was eluted in 50 µl.

RNA (free of genomic DNA by digestion with DNase I) was extracted from 1x10^5 human PBMC or from 200 µl of plasma by using High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer’s instructions. An equivalent volume of culture medium of infected and uninfected cells was included in each batch of RNA extraction as positive and negative controls, respectively. Isolated RNA was eluted in 200 µl.

The concentrations of DNA or RNA were determined by measuring the absorbance at 260 nm (A260) using a spectrophotometer.

*Detection of PERV*
PERV genomes were detected using a novel q-real-time PCR assay and the previously developed PCR ELISA assay (33). Primers and probes of both assays were selected from the cloned PERV insert in p-PCR-PERV.

**PERV q-real-time PCR assay**

Primers and probe used in q-real-time PCR assay were PERV 1-F, PERV 2-R, which amplifies a fragment of 95 bp, and PERV-TM probe (Table1). The q-real-time PCR assay was performed using LightCycler® FastStart DNA Master HybProbe reaction mixture and LightCycler® 2.0 instrument (Roche Diagnostics, Mannheim, Germany). PERV DNA PCR was performed in a 20 µl reaction volume consisting of 15 µl PCR mixture containing 70 nM MgCl₂, 10 pM of each primer, 5 pM Taqman probe and 5 µl of purified DNA (final concentration: 1.5-2.0 ng/µl). The thermal cycling parameters were as follows: initial enzyme activation at 95°C for 8 min, followed by 45 cycles amplification of 5 sec at 95°C, 15 sec at 60°C, 1 sec at 72°C, with a final step of 30 sec at 72°C and 5 sec at 40°C. Each assay included PERV positive and negative controls. Five microliters of each dilution of PERV plasmid DNA were simultaneously assessed to generate a standard curve.

Total cDNA was synthesized with random primers by using Transcriptor High Fidelity cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. Two microliter of total RNA from PBMC or plasma samples were added to RT mixture in a final volume of 20 µl. Thermal cycling parameters for cDNA synthesis were as follows: an initial step at 65°C for 10 min of random hexamer primer and isolated RNA, followed by addition of RT mixture and incubation at 55°C for 30 min, with a final step at 85°C for 5 min.

Two microliters of cDNA were added to the amplification mixture in a final volume of 20 microliters and the PCR was performed as described above.

**Internal controls**

Absence of inhibitors and efficiency of PCR were evaluated by amplification and detection of a 110 bp fragment of the human β-globin gene using LightCycler® Control Kit DNA (Roche Diagnostics, Mannheim, Germany). Amplification and detection of 322 bp fragment of an *in vitro* transcribed
cytochime RNA was used as internal control in RT-PCR assay by LightCycler® Control Kit RNA (Roche Diagnostics, Mannheim, Germany). Amplification reactions of the internal controls and samples were performed simultaneously.

**PCR and RT-PCR ELISA assay**

PCR and RT-PCR ELISA assays were performed as previously reported (33) using PERV 1-F, PERV 1-R primers and biotin-conjugated PERV-BI probe (Table1). In the present study thermal cycling conditions of PCR were optimized as follows: 5 min at 95 °C, then 35 cycles of 20 sec at 94 °C, 20 sec at 55 °C, 45 sec at 72 °C and finally 10 min at 72°C.
Results

Validation of the q-real-time PCR assay

Primers PERV 1-F and PERV 2-R amplify a 95 bp amplicon internal to 151 bp amplicon of the plasmid PCR-PERV. Both amplicons showed a 100% of identity within PERV A, PERV B and PERV C pol gene sequences by using BLAST algorithm. Amplicons produced in the PCR reaction with PERV plasmid and PERV positive controls had the same melting temperature, certifying the specificity of the assay.

A standard curve was generated by 10-fold serial dilutions of PERV plasmid DNA containing from $5 \times 10^6$ to $5 \times 10^0$ copies/reaction mixture. A copy number of $5 \times 10^6$ yielded an average threshold cycle ($C_t$) value of 33.95, whereas a copy number of $5 \times 10^0$ yielded a $C_t$ value of 15.32. Every 10-fold decrease in the copy number yielded an average increase of 3.10 in $C_t$ value. The linearity was obtained for the entire dilution range from $5 \times 10^0$ to $5 \times 10^6$ copies: slope -3.10, intercept 33.95, error 0.00200, efficiency 2.075, correlation coefficient -0.67041.

To test the sensitivity of this method, triplicate serial dilutions of PERV plasmid DNA were added to DNA extracted from $1 \times 10^5$ PBMCs of healthy donors and tested by q-real-time PCR assay.

Eight concentration levels at approximately 1000, 200, 100, 50, 20, 10, 5, and 1 copies of PERV plasmid DNA were tested. The lowest concentration at which detection of PERV plasmid DNA occurred in $\geq 95\%$ of replicates was used to define the detection limit of the assay.

Q-real-time PCR assay showed that 100% of replicates were positive up to 1 copy of PERV plasmid DNA in 5 µl of DNA solution or 10 copies in 50 µl of DNA solution corresponding to the total DNA extracted from $1 \times 10^5$ PBMCs. The amplification curve of one of three replicates is shown in Fig 3. The mean threshold cycles and standard deviations are shown in Table 2. Standard curve assigned a mean concentration of 0.5 copies to the replicates containing approximately one copy of p-PCR-PERV in 5 µl of human DNA solution. We concluded that our assay was capable to reliably detect $\geq 5$ copies of PERV DNA /total DNA isolated from $1 \times 10^5$ PBMCs.

Patient follow-up
Eight living patients, who were treated with the AMC-BAL and liver transplanted, have been followed. In no patient any clinical complication or sign of an unknown infection occurred. In addition the values of biochemical parameters were not different from other liver transplanted patients not receiving AMC BAL treatment. In all treated patients no PERV infection was detected (Fig. 4). PERV DNA and RNA in PBMCs and plasma samples were negative at any points of follow up by using both q-real time PCR (Table 3) and PCR ELISA assay (not shown). The absence of PERV genomes was also found in frozen PBMCs and plasma samples from the patient who died two years after treatment because of a second organ rejection. The amplification curves of q-real-time PCR assay from PBMCs in the last follow up are shown in Fig. 4. Characteristics of patients, durations of follow-up and results of PERV assays are shown in Table 3. Absence of PERV genomes was found in PBMCs and plasma samples from all HCWs included in this study. False negative results were excluded by amplification reactions of the internal controls, done in parallel, with samples. An efficient amplification reaction of internal controls has been obtained in all samples either from patients or HCWs.

Q-real time PCR assays confirmed previous results of PCR ELISA in patient’s plasma samples and PBMCs collected immediately after the BAL-treatment (33). At that time plasma samples were positive for PERV DNA, while PERV RNA was negative. Moreover PBMCs samples were negative for both PERV DNA and RNA. PERV DNA became negative in plasma samples at the first point of follow up occurring 15 to 30 days after BAL treatment.

The detection of PERV DNA in plasma samples from patients at the end of treatment is most likely explained by plasma contamination with genetic material coming from lysis of porcine hepatocytes in the bioreactor during treatment.
Discussion

The clinical use of porcine liver cells in bioartificial liver assist devices is still forbidden in many European countries. Although porcine liver cells remain outside the body of the patient and only have contact with plasma of the patient and not with his immune competent cells, authorities still consider it as a kind of xenotransplantation.

Even if an eventually escape of cells out of the bioreactor to the patient’s circulation is anticipated by cell catching filters in the inflow tract to the patient, extracorporeal porcine-cell-based BALs remain forbidden (29,30). There is good evidence that PERV can be transmitted from cell to cell in vitro and in vivo (9-13), but many publications have shown now that in patients, in the absence of cell-cell contact, PERV transmission does not occur (20-28).

Recently it has been shown (34) that primary human hepatocytes and endothelial cells in culture were reproducibly infected with PERV by supernatants of porcine liver cells which were serum-free cultured in collagen gel for 20 days. Probably these results are due to the fact that porcine cells were cultured in serum-free medium. Previously we and others have argued (35-37) that human plasma comprises natural antibodies against the carbohydrate epitope Gal-α (1-3) Gal, causing immune mediated rejection of pig cells/organs, but also inactivates PERV through complement activation.

The prerequisite of PERV transmission in patients treated with artificial bioreactors based on porcine hepatocytes is the release of infectious PERV from the bioreactor. The virus might cross the membrane filters of the system and might infect the patient. A productive infection with viraemia might occur or a latent infection with integration of PERV genome into host DNA. The latent infection might become productive and even infective in long term immunosuppressed patients.

Since the AMC BAL does not allow direct contact between porcine cells loaded in the bioreactor and patient’s blood cells and complement containing plasma is perfused, transmission of PERV seems to be very unlikely.

However, since it is well known that a retrovirus infection may have latency of many years before any symptoms occur, especially in immune compromised patients, it still remained worthwhile to
exclude PERV infection in our patients and HCWs even after many years. Besides the less sensitive PCR ELISA we have used in the past, now a very sensitive q-real-time PCR assay was applied.

The lack of detection of PERV DNA in PBMCs and the absence of PERV RNA in PBMCs and plasma from treated patients up to 8.7 year follow-up, allow us to exclude PERV latent infection.

We now used a q-real-time PCR assay with high sensitivity and specificity, that also confirmed the absence of PERV RNA in the previous stored plasma samples, assessed by the less sensitive conventional PCR ELISA at that time (33). Therefore this study excludes PERV transmission to liver transplanted patients after AMC-BAL treatment and subsequently pharmacological immunosuppression for many years.

We also confirm that the adherence to recommended infection control measures was sufficient to prevent PERV transmission to HCWs exposed to pig organ or cells during preparation and application of the AMC BAL.

Acknowledgement

We are grateful to mrs R.Hoekstra (PhD) for critical reading of the manuscript.
References


Fig. 1

AMC-bioartificial liver

Plasmapheresis system

Patient

Pump 50 ml/min

Plasma reservoir

Filter

Heater

Pump 150 ml/min

AMC-bioreactor

Gas cylinder

Gas outflow
Fig. 3

Amplification Curves

Fluorescence (F380)

Cycles

1x10E3
2x10E2
1x10E2
5x10E1
2x10E1
1x10E1
6x10E0
1x10E0
Negative control
Fig. 4

Amplification Curves

- 1: Patient 1
- 2: Patient 2
- 3: Patient 3
- 4: Patient 4
- 5: Patient 5
- 6: Patient 6
- 7: Patient 7
- 8: Patient 8
- 9: Patient 9
- 10: HER Positive control
- 11: PK15 Positive control
- 12: Negative control

Fluorescence (CT) vs. Cycles
Legends

Fig. 1. The plasmapheresis system

Fig. 2. Schematic drawing of cross and longitudinal sections of the BAL. A: polysulfon housing; B: three dimensional nonwoven; polyester fabric for high density culture; C: hydrophobic polypropylene hollow fiber membranes for oxygen supply and CO₂ removal; D: extracapillary space; E: side ports for gas supply; F: ports for plasma/medium perfusion

Fig. 3. Sensitivity of PERV q-real-time PCR. PERV plasmid DNA was serially diluted into PBMC DNA of healthy donors and tested in triplicate by q-real-time PCR. The final amount of plasmid copy number in the reaction was 1x10³, 2x10², 1x10¹, 2x10¹, 1x10¹, 5x10⁰, 1x10⁰. The limit of sensitivity was 1 copy of PERV DNA/1.5-2.0 ng of DNA, which corresponds to 5 copies of PERV DNA/total DNA extracted from 1x10⁵ PBMCs. The graph shows PCR amplification curves of one of the three replicates. Straight line is negative control.

Fig. 4. Q-real-time PCR assay from PBMCs of patients at the last follow up after AMC BAL treatment (average: 6.8 years). The graph shows PCR amplification curves. Straight lines represent patients samples and negative control. Two curves refer to positive controls (PK15 and HEK293 PERV-infected cells).
Table 1

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(a) GenBank accession number U77599; (b) from primers PERV 1-F and PERV 2-R
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HBV, hepatitis B virus; HAV, hepatitis A virus; WD, Wilson disease; MSP, mushroom poisoning; AFLP, acute fatty failure of pregnancy; (a) At time of treatment; † deceased patient.
Legends

Tab. 1. Primers and probes from PERV pol gene used in the study.

Tab. 2. Performance of q-real-time PCR: sensitivity assay. Mean threshold cycles and standard deviations of amplification curves in three replicates of each PERV plasmid concentration.

Tab. 3. Characteristics of patients treated with AMC BAL, years of follow up and PERV q-real-time PCR assay results.