Original Article

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일단계 핵산 추출을 이용한 사람거대세포바이러스와 돼지내재레트로바이러스의 검출법

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Detection of Human Cytomegalovirus (HCMV) and Porcine Endogenous Retrovirus (PERV) with One Step Extraction Method

Background: Xenotransplantation is thought to be one of the alternative methods to overcome the shortage of human organs for transplantation. Recipients should be immunosuppressed for graft survival, and thus, there is a need for developing diagnostic modality that can detect diverse infections originating from animals and recipients rapidly, in the early stage, and with high sensitivity using small volume of samples. This study was carried out to develop a fast, simple, and robust technique for the preparation of HCMV DNA and PERV RNA using small volume of samples.

Materials and Methods: Nucleic acids were extracted from serially diluted samples with one step extraction method as well as with Qiagen kit. The presence of genomic DNA of human cytomegalovirus (HCMV) and porcine endogenous retrovirus (PERV) was detected by PCR and specific primer set, respectively. RNA of HCMV and PERV was extracted and then detected by RT-PCR and specific primer set, respectively. For absolute quantification of HCMV, standard curve was established by real time PCR.

Results: HCMV DNA and PERV RNA were prepared from culture supernatant and cells for PCR or RT-PCR with one step extraction method. It was possible to extract both the DNA and RNA from the samples in about 20 minutes with one step extraction method in a single tube. HCMV and PERV could also be detected by PCR and one step extraction method, respectively. It was also good with small quantity samples.

Conclusions: One step extraction method is simpler and faster method than other extraction methods when there are two types of DNA and RNA viruses in one sample. From these results, we could see that the one step extraction method could be very useful in detecting HCMV and PERV rapidly from the pig cells or organ transplanted recipients with a small amount of sample.

Key Words: Porcine endogenous retrovirus, Human Cytomegalovirus, Transplantation, One step extraction method, GeneReleaser

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Introduction

Xenotransplantation is one of the solutions for dealing with the shortage of donor organs for allotransplantation (1), but it carries the potential risk of developing zoonosis (2). Porcine endogenous retroviruses (PERVs) are one of the zoonotic microorganisms. PERVs exist in the genomic DNA of all pigs, and three replication-competent subtypes, A, B and C, have been reported (3-6). Some of them can infect human cells, integrate their genomes into human chromosomes (7), and replicate in a variety of human cells. Immunosuppression is the prerequisite method for successful engraftment. The control of infectious diseases is one of the important elements in transplantation (8). Among them, controlling the human cytomegalovirus (HCMV) infection can be said to be the most important in the management of the transplant recipients who are immunosuppressed (8). These transplant recipients should be mornitored regularly with blood samples among many work-up items. There is a need for developing diagnostic modality that can detect many infectious agents rapidly, in the early stage, and with high sensitivity using small volume of diverse samples. At present, many extraction kits for DNA and RNA are commercially available. Adoption of detection method with a fast, simple, and robust technique is therefore needed to monitor the transplant recipient and to investigate the harm done by HCMV and PERVs to human. This study was performed to compare the fast and simple techniques for preparing HCMV DNA and PERV RNA at the same time.

Methods and Materials

1. Cell lines and viruses

Porcine cell line, PK-15 (ATCC CCL-33), and fetal lung fibroblast (FLF), were cultured in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37℃ in 5% CO_2 incubator. HCMV Towne (ATCC VR-977) was used to infect FLF cells at multiplicity of infection (m.o.i) of 0.01.

2. DNA and RNA Extraction

Two million cells of FLF were infected with HCMV. After 4 days, they were harvested and then resuspended with 66 μL TE buffer; 22 μL were used for extracting DNA by QIAamp DNA mini kit (Qiagen, Hilden, Germany), the other 22 μL for extracting RNA by RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and the remaining 22 μL for extracting both DNA and RNA by one step extraction method. We also extracted DNA and RNA from 22 μL of culture supernatant of HCMV-infected FLF and PK-15 with one step extraction method and QIAamp MinElute media kit. One step extraction method was performed as follows. Briefly, 20 μL of each sample was mixed with 60 μL of resuspended GeneReleaser (Bioventures, USA). After vortexing briefly, tubes containing the mixture were heated at 80℃ for 10 minutes in thermocycler and centrifuged for 10 minutes at ×16,000 g. The supernatant was collected carefully as the source of nucleic acid extraction. Nucleic acids were extracted with phenol for further experiment followed by standard molecular cloning protocols (9).

3. cDNA synthesis

After mixing RNA from each method with primer p(dT)10 (Roche, Germany), the mixture was incubated at 70℃ for 5 minutes and then placed on ice. AccuPower RT premix (Bioneer, Seoul, Korea) was used for cDNA synthesis. Reverse transcription polymerase chain reaction (RT-PCR) was performed. For cDNA synthesis one cycle was performed at 42℃ for 60 minutes; RTase inactivation step was carried out one cycle at 94℃ for 5 minutes.

4. Polymerase chain reaction (PCR)

After the extraction, PCR reactions (Bio-Rad, USA) were carried out in AccuPower PCR premix (Bioneer, Seoul, Korea) containing 10 pmol of forward and reverse primers of HCMV or PERV and varying concentrations of HCMV DNA or PERV cDNA in a total volume of 20 μL. PCR was performed with 40 cycles of denaturation for 10 seconds at 95℃ followed by annealing and extension for 60 seconds at 60℃. The products were then separated using 2% agarose gel electrophoresis (SeaKem, USA). Forward and reverse primer sequences specific for HCMV were located within HCMV US17 and IE1 region. The primers for US17 were 5′-GCG TGC TTT TTA GCC TCT GCA-3′ and 5′-AAA AGT TTG TGC CCC AAC GGT A-3′, and that for IE1 were 5'-TGA CCG AGG ATT GCA ACG A-3' and 5'-AATCGATACCGGCATGATTGA-3'. (10, 11). Forward and reverse primer sequences specific for PERV were located within gag and consisted of 5′-TTG TGT GTC CTT GTC TAC AG -3′ and 5′-TTT TTC TCT CCA AG AGC CAG-3′, respectively.

5. Real time PCR

Real time PCR reactions (ABI PRISM 7900 sequence detection system, Appliedbiosystems, USA) were carried out in Taqman MasterMix (Appliedbiosystems, USA) containing 10 pmol of HCMV forward and reverse primers, 10 pmol of HCMV Taqman probe, and varying concentrations of HCMV DNA in a volume of 20 μL. Real time PCR was performed to quantitate HCMV DNA in samples with the HCMV US17 specific primers and Taqman probe. Taqman probe was synthesized commercially (Bionics, Seoul, Korea) and its sequence was also located within HCMV US

17 region and consisted of 5′-FAM-TGA TCG GCG TTA TCG CGT TCT TGA TC-TAMRA-3′ (10, 11). In case of one step extraction method, it had to be optimized through 8 fold dilution of extracted samples. Real time PCR was performed with universal temperature which is recommended by Appliedbiosystems as follows; 50 cycles of denaturation for 10 seconds at 95℃ was followed by annealing and extension for 60 seconds at 60℃.

Results

1. Quantity and quality of extracted nucleic acid

With one step extraction method, a total of 1.868 μg with 1.802 of 260/280 nm ratio was harvested from 2×10^5 FLF infected with HCMV, as compared with 1.705 μg and 1.815 of 260/280 nm ratio with phenol extraction. The purity of extracted DNA was almost the same in both methods (Table 1).

2. Polymerase chain reaction (PCR)

Capability and specificity of PCR product of DNA, prepared from serially diluted culture supernatants of HCMV infected FLF and PK-15 cells, were verified by 2% agarose gel electrophoresis. One of the data showed that the band of PCR could still be detected in the 10^3-fold diluted culture supernatant of FLF infected with HCMV by one step extraction method, but could hardly be detected by phenol extraction as shown in Fig. 1. The other data showed that the sensitivity of PCR by phenol extraction was 10-fold lower than the sensitivity by one step extraction method as shown in Fig. 2. As depicted in Fig. 3, HCMV could be detected with PCR in 10^4-fold diluted HCMV infected-FLF with Qiagen kit, while it could not be detected with PCR at 1 and 10^4-fold diluted ones with one step extraction method.

3. Reverse transcription polymerase chain reaction (RT-PCR)

After RNA extraction from HCMV infected-FLF and culture supernatant from PK-15, cDNA was synthesized and used as target for specific PCR for HCMV IE1 and PERV gag, respectively. In case of RT-PCR for HCMV IE1, the efficiency of one step and Qiagen extraction method was almost the same. Both of them were detected by RT-PCR in 10^4-fold diluted samples (Fig. 4). Also, the RT-PCR for PERV gag was carried out and revealed positive results only in non diluted samples (Fig. 5).

4. Real time PCR

Standard curve was derived from serially diluted FLF infected with HCMV for real time PCR. Each sample contained from 4×10^5 to 4 cells. Real time PCR could detect HCMV DNA extracted from 4 cells by one step extraction method. Also, standard curve with good slope of -3.68265 and r^2 of 0.993185 was obtained (Table

Table 1. Comparison of the Spectrophotometry Results between One Step and Phenol Extraction*

Method	Phenol extraction	One step extraction
Sample volume	20 μL	20 μL
260/280 nm	1.815±0.092	1.802±0.011
Total amount of DNA (μg)	1.705±0.115	1.868±0.031

*Extraction of DNA from $2x10^5$ FLF infected with HCMV.
The value was expressed as mean±standard deviation.

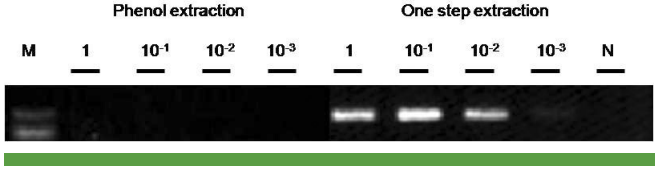


Figure 1. Detection of HCMV DNA for US17 from supernatant of HCMV-infected FLF. The samples were diluted with 10 fold from 1 to 10^{-3} and extracted with phenol and one step extraction methods, and used as templates of PCR. Lane M represents the molecular marker. Lane N represents the PCR results in the absence of templates. The other lanes represent the diluted samples.

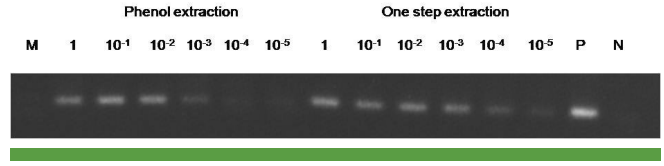


Figure 2. Detection of PERV DNA for gag sequence from PK-15. The samples were diluted with 10 fold from 1 to 10^{-5}, extracted with phenol and one step extraction methods, and used as templates of PCR. Lane M represents the molecular marker. Lane P represents the positive DNA control. Lane N represents the PCR results in the absence of templates. The other lanes represent .the various diluted samples.

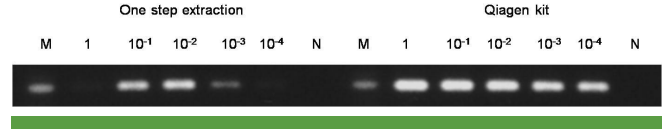


Figure 3. Detection of HCMV DNA for US17 from HCMV-infected fibroblast, diluted with 10 fold from 1 to 10^{-4}, extracted with one step and Qiagen methods, and used as templates of PCR. Lane M represents the molecular marker. Lane N represents the PCR results in the absence of templates. The other lanes represent the various diluted samples.

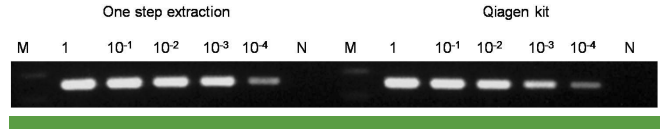


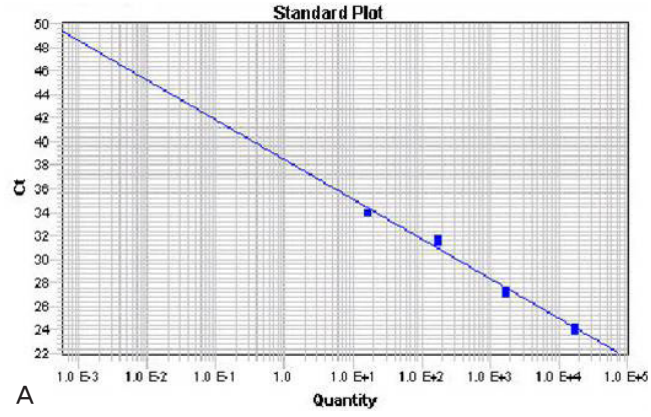
Figure 4. RT-PCR analysis of HCMV infected FLF for HCMV RNA IE1 sequences. RNA of HCMV-infected fibroblast diluted with 10 fold from 1 to 10^{-4}, extracted with one step and Qiagen methods and used as templates of RT-PCR. Then RT-PCR products were used as templates of PCR. Lane M represents the molecular marker. Lane N represents the RT-PCR results in the absence of templates. The other lanes represent the various diluted samples.

Figure 5. RT-PCR analysis of culture supernatant from PK15 for PERV RNA gag sequences. RNA of PK-15 supernatant diluted with 10 fold from 1 to 10^{-4}, extracted with one step and Qiagen methods and used as templates of RT-PCR. Then RT-PCR products were used as templates of PCR. Lane M represents the molecular marker. Lane N represents the RT-PCR results in the absence of templates. The other lanes represent the various diluted samples.

Table 2. Standard Curve Equation of Serially Diluted FLF Infected with HCMV

Slope	Y-Intercept	r^2
-3.68265±0.195772	39.47974±1.336752	0.993185±0.008655

The value was expressed as mean±standard deviation.



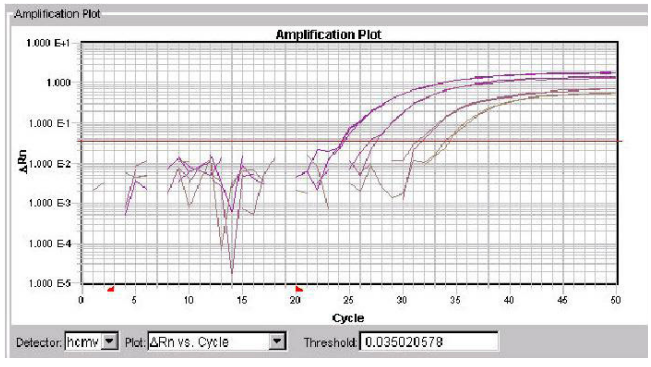


Figure 6. Establishment of quantitative real time PCR. (A) represent a standard curve of serial diluted DNA from HCMV-infected FLF. CT values were expressed in arbitrary units. Logarithms (base 10) were plotted against crossing points. (B) represent a amplification plot.

2). Since the protocol by one step extraction method has been established, it was possible to perform absolute quantification by real time PCR using small amount of samples (Fig. 6).

Discussion

We described one step extraction method for the rapid extraction of nucleic acid, and the detection of HCMV and PERV with the specific amplification of target genes. One step extraction method can be used for detecting DNA of HCMV-infected cells and their culture supernatant. Moreover the one step extraction method can be applied to other viruses including RNA viruses. We have PCR results of PERV which was performed in serially diluted samples. There are two methods for quantitating the genes (12-15). We performed an absolute quantitation of HCMV DNA with real time PCR, with which detecting HCMV DNA extracted from 4 cells by one step extraction method and obtaining good slope and R-square value were possible. This result indicated that the one step extraction method would be very useful in detecting and quantifying HCMV rapidly even in the presence of low concentration of viruses or cells. Phenol extraction took over 4 hours to extract DNA from cells. When using Qiagen kit, specific kit should be applied according to the samples, i.e., cells or supernatant. There are three types of kits for genomic DNA, RNA, and nucleic acid from supernatant. The extraction of genomic DNA and RNA from cells with Qiagen kit took about 30 and 20 minutes, respectively, and that for nucleic acid from supernatant took about 1 hour. In contrast, one step extraction method only took about 15 minutes to extract DNA and RNA from any sample at the same time. In addition, extraction of nucleic acid from supernatant using Qiagen kit required specific equipment. In other words, one step extraction method was simpler, faster, and cost saving method than other extraction methods. One step extraction method also achieves cell lysis, releasing DNA and RNA from the sample in one tube whereas several tubes are used for other methods. Since the experiment is done using the same tube, it could minimize the possibility of cross-contamination which is detrimental in the amplification experiment. However, there are disadvantages with one step extraction method. One step extraction method is powerful for extracting small samples, but it is difficult to extract nucleic acid from large amount of cells or supernatant, is hard to get a high concentration of nucleic acid, and should be diluted to at least 8 folds for efficient PCR. The results of present study showed that one step extraction method could be very useful in detecting HCMV and PERV rapidly from the transplant recipient using a small amount of sample, with minimal chance of cross-contamination.

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