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# Successful removal of porcine circovirus-1 from immunoglobulin G formulated in glycine solution using nanofiltration

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ABSTRACT

Porcine circovirus (PCV) is a potentially harmful virus that has been shown to contaminate biological products. The virus is resistant to many inactivation and/or removal procedures performed during manufacturing. Anion exchange chromatography has been shown to be useful for PCV type 1 (PCV1) removal; however, reduction of PCV1 using methods such as heat inactivation, low pH, nanofiltration, UV-C, and gamma irradiation has not been successful. Therefore, in this study, we evaluate various conditions for process solutions during nanofiltration using PCV1. The results indicated that PCV could be effectively removed from glycine solution at 0.1–0.3 M, pH 4.0 without IgG, using a nanofilter with a pore size of 19 nm (19-nm filter); log reduction values (LRVs) of $\geq$4.5 and $\geq$ 5.0, respectively, were obtained. In contrast, PCV1 was significantly removed (LRV: 2.2) in glycine solution at 0.3 M, pH 6.0 with 1.0% IgG using the 19-nm filter, but some virus genomes were detected in the filtrates. In summary, the use of a 19-nm filter in glycine solution with/without IgG is an appropriate condition for PCV removal.

## 1. Introduction

In the biopharmaceutical industry, animal cells and animal-derived raw materials, serum and enzymes such as trypsin and pepsin are often used during the manufacturing process. Therefore, there is a risk of viral contamination, and safety measures and evaluations are necessary to provide a sufficient margin of safety [1,2]. In recent years, contamination with small viruses, such as cache valley virus, human adenovirus, minute virus of mice (MVM), and porcine circovirus (PCV), has been reported [3–9]. DNA from PCV type 1 (PCV1), a very small virus, was detected in the human vaccine Rotarix (GlaxoSmithKline, Rixenxart, Belgium), and small fragments of PCV1 and PCV type2 (PCV2) DNA were also detected in RotaTeq (Merck and Co., West Point, PA, USA) [7–9]. The source of contamination was considered to be a lot of trypsin derived from swine.

PCV, which belongs to the family *Circoviridae*, has a small nonenveloped virus capsid and contains a single-stranded circular DNA genome of 1.76 kb. The viral particle has a diameter of approximately 17–20.7 nm [10]. PCV1 infection is common in swine, and the virus has not been shown to have pathogenicity in swine or humans [11,12]. In contrast, PCV2 induces postweaning multisystemic wasting syndrome in swine, but has still not been shown to have pathogenicity in humans [13]. Notably, in addition to trypsin-derived PCV contamination, PCV DNA has also been detected from commercial pepsin products derived from swine [14]. An unexpected persistent infection of PCV1 was found in the established pig kidney cell line PK-15 [15]. PCV is extremely resistant to physicochemical inactivation procedures, such as heat treatment, low pH, UV-C, gamma irradiation, and virus filtration [16–20]. Therefore, PCV has the potential to cause contamination via source materials derived from swine.

To reduce the risk of viral contamination in final products, effective methods for removal of PCV during the manufacturing of biological products are required. A strong anion exchange chromatographic resin (Q sepharose fast flow) has been shown to have good capacity for removal of PCV during the manufacturing of monoclonal antibodies [18]. Other methods for effective removal or inactivation of PCV have not yet been proposed. In a previous study, human parvovirus B19 in 0.3 M glycine solution, pH 6 was aggregated, and a high extent of removal was observed [21]. The aggregation is influenced by solution composition and pH and the isoelectric point of the virus particle. Therefore, it is still unknown whether glycine has potential for PCV aggregation. Combining different mechanisms of virus inactivation or removal may

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be effective for virus elimination during manufacturing [1]. Accordingly, in the present study, we evaluated conditions for effective removal of PCV by nanofiltration using glycine to develop methods for protection against PCV contamination.

## 2. Materials and methods

### 2.1. Cells, viruses, and samples

PCV1 was collected from culture supernatants of PK-15 cells (cat. no. CCL-33; American Type Culture Collection [ATCC], Manassas, VA, USA). Culture supernatants of PK-15 cells after 4 days of cultivation with Dulbecco's modified Eagle's medium (DMEM; Invitrogen/Thermo Fisher Scientific Inc., MA, USA) containing 10% fetal bovine serum (FBS; HyClone/GE Healthcare UK Ltd., Little Chalfont, UK) were collected and stored at −80 °C as a PCV1 stock. The amount of PCV1 genome in the stock was 10.17 $\log_{10}$ copies/mL, as determined by quantitative polymerase chain reaction (qPCR). MVM (cat. no. VR-1346; ATCC) and A9 cells (cat. no. CCL1.4; ATCC) were also used in this study. The MVM stock was prepared by postinfection cultivation with DMEM containing 1% FBS for 12–15 days as described above. The infectivity of the MVM stock was 6.8 $\log_{10}$ tissue culture infective dose ($TCID_{50}$)/mL, as determined by infectivity assays. PCV1 and MVM stocks were ultracentrifuged at 150,000 × *g* for 3 h at 4 °C, and the precipitates were resuspended in phosphate-buffered saline (PBS) at a volume equal to the volume before centrifugation, filtered with a 0.22-μm filter and stored at −80 °C for use as a spike source. Each virus stock was thawed at 37 °C and sonicated followed by 0.22-μm filtration before spiking. A polyclonal immunoglobulin derived from human plasma (IVIG; Venoglobulin-IH; Japan Blood Products Organization [JB], Tokyo, Japan) was also used as a test material. The nanofilters Planova 15N (15-nm filter; mean pore size of 15 ± 2 nm, 0.001 $m^2$; Asahi Kasei Medical Co., Ltd. Tokyo, Japan), Planova 20N (19-nm filter; 19 ± 2 nm, 0.001 $m^2$; Asahi Kasei Medical), and Planova 35N (35-nm filter; 35 ± 2 nm, 0.001 $m^2$; Asahi Kasei Medical) were used for virus filtration in this study.

### 2.2. Determination of the amount of PCV1 genome by qPCR

The amount of PCV1 genome in the sample was determined using an Applied Biosystems 7500 real-time PCR system (Applied Biosystems/Thermo Fisher Scientific Inc.). Briefly, the samples were diluted with PBS, and PCV1 DNA was extracted using a QIAamp viral RNA Mini QIAcube Kit (Qiagen GmbH, Hilden, Germany). PCV1 DNA was quantified by qPCR using the following primer/probe set: forward, 5′-AGAAAGGCGGGAATTGAAGATAC-3′, reverse, 5′-CACACCCCGCCTTCAGAA-3′, and TaqMan probe, 5′-CGTCTTTCGGCGCCATCTGTAACG-3′ containing FAM and TAMRA tags (Applied Biosystems) [18]. A synthetic control PCV1 DNA template was used as a standard (Sigma-Aldrich Japan K·K., Tokyo, Japan). The PCR amplification was performed at 95 °C for 15 min, followed by 40 cycles at 94 °C for 15 s and 60 °C for 1 min.

### 2.3. Infectivity assay for MVM

A9 cells were cultured in DMEM supplemented with 10% FBS. The cells were grown to subconfluence in 96-well plates and then inoculated with 10-fold serial dilutions of the test sample (0.03 mL/well). After incubation at 37 °C for 60 min, 0.075 mL of medium with 1% FBS was added to each well, and cells were cultured at 37 °C in an atmosphere containing 5% $CO_2$. At 2 days postinfection, cells were washed, and the medium was replaced with fresh medium. Cells were then incubated for an additional 5 days. The cytopathic effect (CPE) was examined, and the MVM infectivity titer was determined by the Spearman-Kärber method.

### 2.4. Effects of pH and glycine concentration on PCV1 removal by nanofiltration

To evaluate virus removal ability during virus filtration by pH, PCV1 was spiked (1/100 v/v) into each 0.3 M glycine solution at pH 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0, and 30-mL samples were subjected to virus removal filtration using 15-nm, 19-nm, and 35-nm filters. The filtrations were performed under conditions of less than 80 kPa, dead-end mode, and room temperature. PCV1 was also spiked (1/100 v/v) into each pH 4.0 glycine solution at concentrations of 0, 0.1, 0.3, 0.5, and 2.0 M to evaluate virus removal according to glycine concentration. The spiked samples were subjected to virus removal filtration (30-mL/0.001 $m^2$, 80 kPa at room temperature), as described above. The amounts of PCV1 DNA before and after filtration were determined by qPCR, as described above. Log reduction values (LRVs) were calculated as the amount of total virus before and after filtration.

### 2.5. Interference by the presence of IgG during PCV1 removal by nanofiltration

To clarify the appropriate pH conditions for PCV1 removal in the presence of IgG, PCV1 was spiked (1/100 v/v) into 0.1 M glycine solution containing 0.1% IgG at pH 3.5, 4.0, 4.5, 5.0, and 6.0 to evaluate virus removal according to pH. Virus removal filtration was performed as described above. The effective glycine concentration for PCV1 removal in the presence of IgG was then determined by spiking PCV1 (1/100 v/v) into 0.1% IgG in glycine solutions (0.1, 0.3, or 0.5 M; pH 6.0), and virus removal filtration was performed as described above. The amounts of PCV1 DNA before and after filtration were determined by qPCR, as described above.

### 2.6. Effective conditions for PCV1 removal by nanofiltration

To clarify the effective conditions for a range of IgG concentrations, PCV1 was spiked (1/100 v/v) into 0.3 M glycine solutions (pH 6.0) containing IgG (0.1%, 0.5%, 1.0%, 1.5%, 2.0%, or 5.0%). In addition, we also evaluated 0.1 M glycine solution (pH 4.0) containing IgG (0.1%, 0.5%, or 1.0%). Virus removal filtration was performed as described above. The amounts of PCV1 DNA before and after filtration were determined by qPCR. Then, to clarify the IgG recovery during filtration, filtration of 1.0% IgG in glycine solution (0.3 M, pH 6.0) was performed using the 19-nm filter. Protein concentrations in samples before and after filtration were measured by UV-C 1700 (Shimadzu Corporation, Kyoto, Japan) at 280 nm, and the recovery rate was calculated. MVM was also spiked (1/100 v/v) into the 0.3 M glycine solution containing 1.0% IgG (pH 6.0), and a 30-mL sample was subjected to virus removal filtration using the 19-nm filter. The infectivity titers of MVM before and after filtrations were determined by infectivity assay.

## 3. Results

### 3.1. Effects of pH and glycine concentration on PCV1 removal by nanofiltration

To evaluate virus removal during virus filtration by pH, PCV1 in 0.3 M glycine solution with different pH values was examined. The PCV1 genome was not detected in the filtrates of 15-nm and 19-nm filters at pH 4.0, and the LRVs of each filter were ≥5.0; in contrast, the genome was detected in the filtrate of the 35-nm filter at pH 4.0, and the LRV was 3.8. These results suggested that the most effective condition for PCV1 removal was pH 4.0 (Fig. 1). Then, to evaluate virus removal during virus filtration according to changes in glycine concentrations, PCV1 in various concentrations of glycine solution at pH 4.0 was also examined. The PCV1 genome was not detected in the filtrates of 15-nm and 19-nm filters at concentrations of 0.1 and 0.3 M.