Porcine Circovirus (PCV) Removal by Q Sepharose Fast Flow Chromatography

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The recently discovered contamination of oral rotavirus vaccines led to exposure of millions of infants to porcine circovirus (PCV). PCV was not detected by conventional virus screening tests. Regulatory agencies expect exclusion of adventitious viruses from biological products. Therefore, methods for inactivation/removal of viruses have to be implemented as an additional safety barrier whenever feasible. However, inactivation or removal of PCV is difficult. PCV is highly resistant to widely used physicochemical inactivation procedures. Circoviruses such as PCV are the smallest viruses known and are not expected to be effectively removed by currently-used virus filters due to the small size of the circovirus particles. Anion exchange chromatography such as Q Sepharose® Fast Flow (QSFF) has been shown to effectively remove a range of viruses including parvoviruses. In this study, we investigated PCV1 removal by virus filtration and by QSFF chromatography. As expected, PCV1 could not be effectively removed by virus filtration. However, PCV1 could be effectively removed by QSFF as used during the purification of monoclonal antibodies (mAbs) and a log_{10} reduction value (LRV) of 4.12 was obtained. © 2013 American Institute of Chemical Engineers Biotechnol. Prog., 29:1464–1471, 2013
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Introduction

Two attenuated live rotavirus vaccines, Rotarix (GlaxoSmithKline, Belgium) and RotaTeq (Merck and Co.), are being used worldwide to prevent rotavirus gastroenteritis in children. They are orally administered to infants, with the vaccination schedule starting at a very early age. Porcine circovirus type 1 (PCV1) sequence was first reported in Rotarix by a metagenomic analysis.[1] The contamination of oral rotavirus vaccines led to exposure of millions of infants to PCV1.[2] Since then, extensive efforts from vaccine manufacturers, academic institutes, and regulatory agencies (the US Food and Drug Adminstration [FDA], the Europe Medicines Agency [EMA], National Institute for Biological Standards and Control [NIBSC] and the Paul-Ehrlich-Institute, Germany) have been made to investigate the virus contamination in vaccines. Polymerase chain reaction (PCR) analysis revealed both PCV1 and PCV2 (Porcine circovirus type 2) DNA in RotaTeq (Merck and Co., West Point, PA). Investigations by the vaccine manufacturer and the US FDA independently demonstrated infectious virus in Rotarix, although infectious virus was not demonstrated in RotaTeq.[3]

PCVs are the smallest known non-enveloped mammalian viruses that contain a single-stranded, circular DNA genome and are present in swineherds throughout the world. Two PCVs (PCV1 and PCV2) have been identified so far. PCV1 is not known to cause any disease in animals, but PCV2 has been identified as the causative agent of post-weaning multi-systemic wasting syndrome (PMWS). In addition to human vaccines, PCV1 contamination has previously been detected in commercial pig vaccines using PCR.[4] Another study also showed that PCV was detected from a porcine-derived commercial pepsin product that may potentially be used in biological manufacture.[5] While PCV contamination of these medicinal products was not investigated in the following cases, porcine-derived FVIII has been shown to be contaminated with porcine parvovirus (PPV).[6] In addition, porcine HoKo-virus (i.e., PARV4-like porcine parvovirus) was detected in such preparations as well.[7] The recent contamination of PCV DNA in rotavirus vaccine was found to be most likely to originate from animal-derived trypsin introduced in vaccine production. Porcine trypsin is widely used in biotechnology industry in cell culture process to detach cells or to process recombinant insulin used as cell culture ingredient. Porcine trypsin is also an activator of rotavirus and influenza virus vaccines. A recent study by FDA also raised concern for PCV contamination of production cell lines, emphasizing the need for continued efforts to reduce the likelihood of

introducing viruses such as PCV from animal-derived raw materials used in biological product manufacture.[8] Although there is no evidence that PCV causes disease in humans, the expectation from regulatory agencies is that biological products should be free of adventitious viruses. The safety of biological products relies on the implementation of three rigorous complementary approaches to mitigate potential contamination by viruses: (1) selection and testing of cell lines and other raw material for the absence of viruses; (2) testing the product at appropriate steps during production, to confirm the absence of contaminating infectious viruses; and (3) assessing the capacity of the production processes to inactivate/remove viruses.[9–11] In the case of vaccine contamination by PCV, the virus was undetected by the commonly used virus testing methods because it does not cause obvious cytopathic effects (CPE) in *in vitro* cell culture, hemagglutination, hemadsorption, or symptoms of infection in animals. Therefore, demonstration of the capacity of the production processes to clear (remove or inactivate) viruses becomes a very important complementary method to maximize virus safety. PCV is highly resistant to widely used physicochemical inactivation procedures such as low pH treatment, heat treatment, gamma or UV irradiation.[12–14] Moreover, since PCV is a virus even smaller than members of the parvovirus family, effective removal of PCV by commercially available filters, which have been designed for the removal of parvoviruses, has not been validated. Therefore, an alternative method for the effective removal of PCV is urgently desired.

Anion exchange (AEX) chromatography procedures have been shown to remove many biological impurities, including viruses, during the purification of mAb and are regularly used during manufacturing.[15–17] Virus reduction studies investigating AEX chromatography procedures have shown such steps to be highly effective in removing viruses, consistently achieving $\log_{10}$ reduction values (LRVs) greater than 4.[18–26] More recent study also demonstrated that AEX Q membrane efficiently removed different model viruses in a range of operational parameters[27]. The AEX process used to purify many mAbs is straightforward in mAb flow-through mode. Due to the high isoelectric point of the mAbs, buffer conditions are generally chosen so that the antibody flows through the column or membrane, while impurities such as viruses are retained.[15,27] The binding of those impurities is believed to occur through an electrostatic interaction with the anion exchange media. Among AEX, QSFF chromatography has been the most extensively characterized for its virus clearance capacity. The mechanism of virus removal by QSFF is believed to be similar to that of other impurity removal by QSFF resin, where mAbs with high isoelectric points flow through the column but a wide range of viruses with low isoelectric points bind to the resin.[28] The robust mAb flow-through QSFF process has been shown to be capable of effectively removing many viruses with various biochemical and biophysical properties (enveloped to non-enveloped viruses of differing sizes, included parvoviruses, one of the smallest viruses used in reduction studies). Our previous study also showed that QSFF process effectively removed both in-process and spiked retrovirus-like particles (RVLPs) that are expressed during the production of mAbs in Chinese hamster ovary (CHO) cell cultures.[29] The robust nature of virus removal by QSFF was also investigated using a statistical design-of-experiments (DOE) approach, which showed that varying many process parameters simultaneously did not affect the ability of the QSFF process to remove viruses

including RVLP.[30] In addition, viral clearance capacity by QSFF was maintained after extensive re-use.[20]

In this study, we have evaluated PCV1 removal by virus filtration and by the QSFF product flow-through procedure commonly used in mAb purification to demonstrate the feasibility of PCV1 removal by the resin using quantitative PCR (QPCR) and analysis of virus infectivity. PCV1 virus stock was first characterized to ensure the quality of the virus stock is suitable for study needs. To better characterize the level of PCV1 removal, comparative studies were performed using PCV1 and minute virus of mice (MVM), a widely used, small-size model DNA virus with a well-understood mechanism of removal by QSFF.

Materials and Methods

Virus stocks

The PCV1 isolate from PK-15 cells (GenBank AY193712) was obtained from WuXi-Apptec (St Paul, MN); PCV1 was purified by ultracentrifugation. MVM was obtained from BioReliance (Rockville, MD), with the stock titer at 8.0 to 8.5 $\log_{10}$ $TCID_{50}$/mL.

Real-time quantitative PCR (QPCR)

For the MVM QPCR assay, samples were first subjected to DNaseI (Life Technology, Grand Island, NY) digestion using 20 units per reaction for 20 min at 37°C to remove free DNA. Extraction of virus nucleic acid was then performed using the EZ1 Virus Mini Kit v2.0 on a BioRobot EZ1 (Qiagen, Inc., Valencia, CA). The QPCR assay used to quantify viral particles (VP) was performed as previously described.[31] For virus clearance studies, sample interference was determined by comparing 1:10 diluted samples with undiluted samples.

For PCV1 QPCR assay, samples were first treated with 250 units of Benzonase for 1 h at 37°C with a final concentration of 2 mM $MgCl_2$. Thereafter, DNA was isolated using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) or the High Pure Viral Nucleic Acid Kit (Roche Applied Science, Mannheim, Germany). DNA was eluted in 100 μL (Qiagen) or 50 μL (Roche) elution buffers, respectively and 10 μL of the eluate were subjected to PCR. The QPCR used for the quantification of PCV-1 DNA was performed using primers (PCV-1 TM Fwd.: AGAAAGGCG GGAATTGAAGATAC and PCV-1 TM Rev.: CACACCCC GCCTTCAGAA) and a TaqMan probe (6FAM-CGTCTT TCGGCGCCATCTGTAACG-BBQ) designed with Primer Express 3.0 software (Applied Biosystems, Darmstadt, Germany). A synthetic control DNA template was used as a standard (Amptech, Hamburg, Germany). Amplification was performed using an ABI Prism 79000 HT fast PCR device (Applied Biosystem, Darmstadt, Germany).

MVM infectivity assay

Infectious MVM was quantified by endpoint-titration on NB324K cells determining the tissue culture infectious dose 50 ($TCID_{50}$), i.e., the dose where 50% of cell cultures are infected. NB324K cells were cultured in Eagle's minimal essential medium, 5% fetal bovine serum (FBS), 2 mM L-glutamine. Briefly, cells were grown to sub-confluence in 96-well microtitre plates and inoculated with 50 μL sample from a dilution series of the test item. The samples were

then incubated in 5% CO_2 at 37°C for 7 days and examined for cytopathic effect (CPE). Certain samples were sub-cultured by transferring a small portion of sample into the wells of newly seeded plates and incubated for another 7 days to confirm the CPE. Virus titer was then determined using the Spearman–Karber method. If no virus was detected, the detection limit was calculated by Poisson distribution. Large volume (LV) testing with 4 mL of sample was performed for all of the samples, except for the spiked load, in order to achieve higher assay sensitivity.

PCV1 infectivity assay

Infectious PCV1 was quantified by endpoint-titration using PK13 cells and expressed in $TCID_{50}$. PK13 cells were cultivated in DMEM, 10% FBS, 2 mM L-glutamine. 10^5 PK13 cells were seeded into 24-well plates and infected the following day with 200 μL of pre-diluted samples. Inoculum was removed after 1 h adsorption and 1 mL cell culture medium was added to the cells. The PCV1 DNA concentration in cell culture supernatants was determined by QPCR at day 1 post-infection (one culture per dilution step) and 6 days post-infection (3–5 cultures per dilution step). The infectivity assay was scored positive whenever there was a clear (i.e., more than 1 log_{10}) increase in viral DNA concentration between 1 and 6 days post-infection. Infectivity titers from end point titration were calculated by maximum likelihood algorithm[32] using ClickIT software kindly provided by Baxter.

Transmission electronic microscopy (TEM)

The PCV-1 virus stock was fixed and negatively stained using 2% aqueous uranyl acetate and examined by electron microscopy (Zeiss EM 902, Zeiss, Germany).

Virus filtration

Virus was spiked 1:1000 into cell culture medium (DMEM) and treated with nuclease as described above and the spiked material was pre-filtered through a Minisart 0.2 μm sterile filter (Satorius, Göttingen, Germany). The spiked medium (25 mL) was processed through a 0.001 m^2 Planova 20N filter (Asahi Kasei, Cologne, Germany) and subsequently 20 mL from the collected filtrate was filtered through a 0.001 m^2 Planova 15N filter (Asahi Kasei) in accordance with the manufacturer's instructions. Filtration was performed in "dead-end mode" and pressure did not exceed 1 bar. The filters were integrity tested (for the 15N and 20N filters) after all the process runs.

Anion exchange chromatography

All viral clearance chromatography runs were performed using an ÄKTA Purifier 100 (GE Healthcare, Piscataway, NJ). Small-scale chromatography columns (0.66 cm diameter, Bio-Chem Valve/OmniFit, Boonton, NJ) were packed with naïve QSFF resin to a bed height of 20 cm and were equilibrated with eight column volumes (CV) of equilibration buffer (25 mM Tris, 25 mM NaCl, pH 8.0) at 0.86 mL/min. For each chromatography run, the same buffer was spiked with nuclease (Benzonase) pre-treated 1% v/v of MVM or 0.5% of PCV1 stock solutions and filtered through a 0.22 μm filter (Corning, Corning, NY). The MVM or PCV1-spiked buffer was then loaded onto the column at the same flow rate and two flow-through fractions were collected from the beginning of the load phase to the end of the load phase. Then the column was washed with 3 CV of equilibration buffer and the wash fraction was also collected. After the wash phase, the column was eluted by a 25–300 mM NaCl salt gradient containing 25 mM Tris at pH 8.0. A series of elution fractions were collected every 1 CV. Upon completion of the chromatography run, aliquots of all the collected pools were diluted as necessary, and stored at −80°C. LRVs were calculated as the difference between logarithmic values of total virus load in the spiked column load material and total virus load in the pooled flow-through fractions.

Results and Discussion

Characterization of the PCV1 virus stock

Careful characterization of the PCV1 virus stock is important for interpretation of virus reduction data. For example, aggregated virus particles can be removed better than mono-dispersed particles causing overestimate of virus removal. On another hand, too much free nucleic acid in virus stock may cause nucleic acid penetration of virus filter which would lead to an underestimation of virus reduction. A combination of nuclease treatment and QPCR was used in order to quantify virus particles. QPCR measures virus particles (encapsidated DNA) as well as free virus DNA (non encapsidated DNA released from infected cells). However, free nucleic acid can be removed by nuclease pre-treatment of samples before extraction of DNA from virus capsid. Therefore, we measured the DNA concentration before and after extensive nuclease treatment of the virus preparation to determine the level of free virus DNA in the stock. The DNA concentration of the virus stock preparation was 10.39 log_{10} geq/mL without nuclease treatment and 10.19 log_{10} geq/mL with nuclease treatment indicating that the PCV1 stock mainly represented encapsidated virus particles. PCV1 grows in cell culture without any visible CPE or alteration of cell growth kinetics and viability. Therefore, it is not possibly to identify infected cells by light microscopy in a classical virus titration assay. Endpoint titration was performed using the increase in PCV1 DNA concentration in the cell culture supernatant as a read-out for detection of infected cells. The endpoint concentration was 6.12 ± 0.62 log_{10} $TCID_{50}$/mL. Comparing the infectious titer with nuclease-resistant virus DNA concentration indicated a ratio of one $TCID_{50}$ per 1.2×10^4 virus particles. Electron microscopic examination of the virus stock preparation confirmed that the virus particle concentration was in the same order of magnitude (*ca*. 10^{10} to 10^{11} particles/mL) as expected from QPCR assay and showed mainly monodisperse virus particles with the expected size of 15–20 nm (Figure 1). This indicates PCV1 virus stock is suitable for virus removal studies.

PCV1 removal by virus filtration

The most recognized effective method to remove adventitious viruses is virus filtration. This is particularly true for viruses, such as the small non-enveloped parvoviruses, which are resistant to physicochemical inactivation treatments. Current small virus filtration technology, widely used in biological product manufacture, is considered as the most important state of the art virus clearance technology and is effective for removal of parvoviruses with an average size of
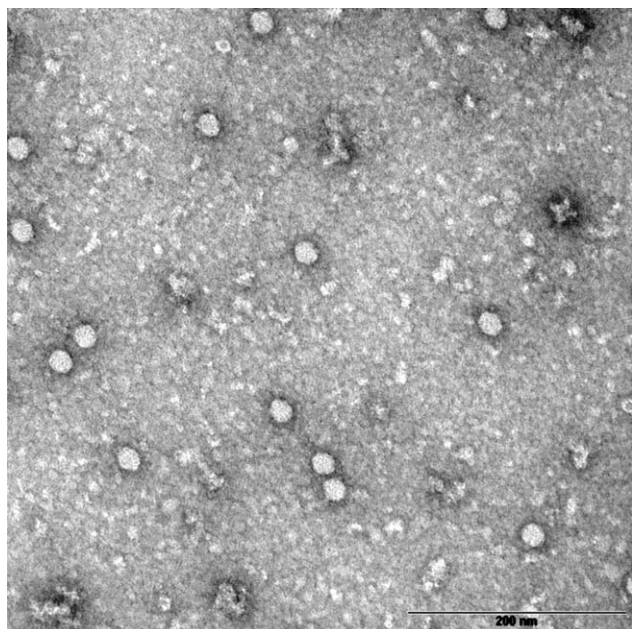
Figure 1. Analysis of purified PCV1 by negative staining and electron microscopy.

Table 1. Virus Reduction Factors ($\log_{10}$) by Serial Virus Filtration

Run	A	B	C	D
Matrix	DMEM*	DMEM*	DMEM*	Albumin‡
0.2 µm Pre-filtration	0.12	0.04	0.03	−0.75
Planova 20N	0.13	0.24	0.72	0.45
Planova 15N	1.77	1.30	1.46	1.47

*DMEM: Dulbecco's modified minimal essential cell culture medium.
†PCV1 spike preparation from PK15 cells.
‡PCV1 spike preparation from PS cells.
§Albumin: 0.5% human serum albumin.

approximately 20 nm. As with all membrane-based filters, virus filters have a distribution of pore sizes. Hence, membrane filters are often described by an average pore size.[33] The average pore size of the Planova 15N filter is somewhat smaller (15 nm) than that of the Planova 20N filter (20 nm). Virus reduction across such filters can be heavily influenced by the aggregation status of virus particles. PCV1 was spiked into cell culture medium or 0.5% albumin model protein solution, as it is known that these matrices do not provoke aggregation of virus particles. First the spiked material was processed through a 0.2 µm sterile filter, then through a Planova 20N filter and subsequently through a Planova 15N filter. The volumetric loads were 25 L/m^2 for the Planova 20N and 20 L/m^2 for the Planova 15N filter, respectively. The virus reduction factors are summarized in Table 1. As expected there was no virus reduction by the 0.2 µm prefilter. The Planova 20N filter did not significantly reduce the virus loads ($\log_{10}$ reduction factors ranging from 0.13 to 0.72 in 4 separate runs). Reduction of PCV1 was observed with the Planova 15N filter, however this reduction was moderate in the order of 1.5 $\log_{10}$ ($\log_{10}$ reduction factors ranging from 1.30 to 1.77 in 4 separate runs). The reduction by the Planova 15N filter is considered significant, but too limited for effective removal of PCV1 from contaminated material.

There was no influence on virus reduction when a 0.5% protein (human serum albumin) containing solution was filtered (run D) instead of protein-free DMEM cell culture medium (runs A, B, and C). Furthermore, using a different virus spike preparation from other cells (PS cells in run C) instead of PK15 cells (runs A, B, and D) did not affect virus reduction. Using other virus retentive filters such as Planova BioEx and Millipore VPro resulted in reduction factors below 1 log10 (data not shown). In summary, these results indicate the limitations of virus filters for reduction of very small circoviruses such as PCV1.

The filtration experiments were in agreement with our expectations from the virus particle size and average pore size of virus filters. The current virus filters which have been designed for removal of parvoviruses are not suitable for effective reduction of smaller viruses such as PCV. While it might be technically feasible to manufacture virus filters with smaller average pore size, it seems questionable whether such filters could be successfully applied in biotechnology, considering the size of many biotherapeutic proteins and the demand on high throughput at large scale manufacture of such proteins.

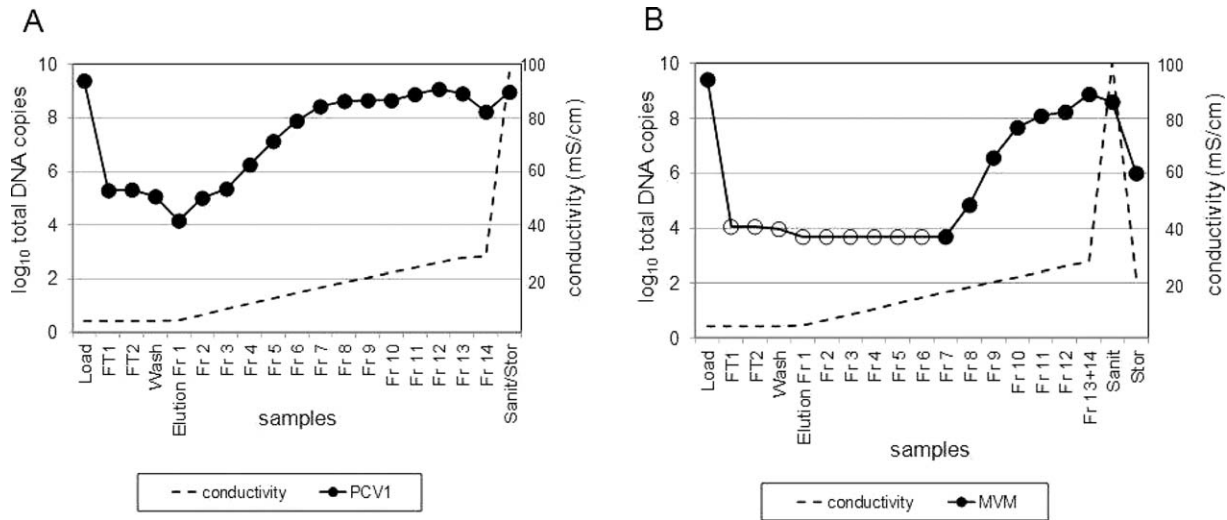### PCV1 and MVM removal by QSFF chromatography

QSFF product flow-through process is often used as one of the chromatography steps during mAb purification. The step is performed under conditions (the feedstock is typically loaded at or near pH 8.0) where mAbs, with high isoelectric points, flow through the column, but viruses with low isoelectric points and other impurities such as host cell proteins and host cell DNA are removed by binding to the resin. We determined removal of PCV1 by using typical QSFF chromatography manufacturing operating conditions at pH 8.0. To simplify data interpretation, the feedstock was only a buffer, at pH 8.0, which did not contain mAb. Feedstock spiked with the PCV1, representing 0.5% of the load volume, produced a virus concentration of $10^{8.13}$ viral particles per mL (Table 2). After processing the PCV1 spiked feedstock over the QSFF column, the flow-through and wash pools were collected and the total number of PCV1 virus particles in the pools was quantified using the QPCR assay. To ensure that only encapsidated virus particles were quantified, virus stocks were pre-treated by Benzonase before column loading. The removal of PCV1 from the flow-through and wash pools was not complete and residual level of 5.28 to 5.32 $\log_{10}$ PCV1 remained in the pools (Table 2). However, a LRV value of 4.12 was obtained, representing an effective PCV1 removal by the process from the flow through and wash pool that would contain the purified antibody during biotechnological production (Table 2). In addition, to evaluate if PCV1 binding to the QSFF resin could be disrupted by high salt and the point at which it elutes, an elution step was performed after the wash phase by using a NaCl gradient of 25–300 mM and 1 CV fractions were collected for PCV1 quantification by QPCR. Table 2 shows that a low level of 4.15 $\log_{10}$ PCV1 virus particles was in the first elution fraction and gradually increased up to 9.08 $\log_{10}$ as NaCl elution concentration increased. The data thus indicated that the QSFF process is capable of effectively removing high levels of PCV1 virus and electrostatic interaction between the virus and the resin may be the primary mechanism of PCV1 removal by QSFF. Table 2 also shows the sum of PCV1 from all the collected elution fractions was almost identical to the amount of PCV1 virus loaded on the column, suggesting all loaded PCV1 was eluted from the resin by high salt.

Table 2. PCV1 Distribution and Removal During QSFF Chromatography

Sample	Cond. (mS/cm)	Volume (mL)	DNA ($\log_{10}$ GE/mL)	Total DNA ($\log_{10}$)	Infectivity ($\log_{10}$ TCID$_{50}$/mL)	Total infectivity ($\log_{10}$ TCID$_{50}$)
Load	4.2	50.4	8.13	9.83	2.55 ± 0.87	4.25
Flow through 1	4.2	25.0	3.88	5.28	<1.13	<2.53
Flow through 2	4.2	25.0	3.92	5.32	<1.13	<2.53
Wash	4.2	20.5	3.76	5.07	<1.13	<2.44
Elution Fr. 1	4.5	10.3	3.14	4.15	<1.13	<2.14
Elution Fr. 2	6.5	10.3	3.99	5.00	<1.13	<2.14
Elution Fr. 3	8.6	10.3	4.34	5.35	<1.13	<2.14
Elution Fr. 4	10.7	10.3	5.23	6.24	<1.13	<2.14
Elution Fr. 5	12.6	10.3	6.11	7.12	<1.13	<2.14
Elution Fr. 6	14.6	10.3	6.88	7.89	<1.13	<2.14
Elution Fr. 7	16.5	10.3	7.42	8.43	1.75 ± 0.65	2.76
Elution Fr. 8	18.5	10.3	7.62	8.63	1.75 ± 0.65	2.76
Elution Fr. 9	20.3	10.3	7.65	8.66	2.72 ± 0.64	3.73
Elution Fr. 10	22.2	10.3	7.63	8.64	1.83 ± 0.67	2.84
Elution Fr. 11	24.0	10.3	7.88	8.89	2.72 ± 0.64	3.73
Elution Fr. 12	25.9	10.3	8.07	9.08	2.72 ± 0.64	3.73
Elution Fr. 13	27.7	10.3	7.91	8.92	2.52 ± 0.64	3.53
Elution Fr. 14	28.3	3.1	7.75	8.24	<2.31	<2.80
Sanit/Stor	97.1	78.7	7.05	8.95	<2.13	<4.03
LRV				4.12*		$\geq$1.27*
						$\geq$3.49†

*logarithmic ($\log_{10}$) reduction factor (LRV) calculated from total virus loads in Load-fraction and combined fractions "Flow through 1, Flow through 2, and Wash".

†LRV determined from re-titration of large volume samples where total infectivity in load-fraction was 5.77 $\log_{10}$ and total infectivity in combined fractions "Flow through 1, Flow through 2, and Wash was $\leq$2.28 $\log_{10}$.

Figure 2. Removal and Elution of PCV1 and MVM during QSFF Chromatography.

(A) Total PCV DNA copies in load, flow through pools, wash pool, and a series of elution fractions generated by a linear NaCl gradient (25–300 mM). (B) Total MVM copies in load, flow through pools, wash pool, and a series of elution fractions by a linear NaCl gradient (25–300 mM). Open circles indicate negative DNA assay below limit of quantification.

PCV1 removal by the QSFF chromatography process and its elution pattern were compared to the removal of MVM which is well understood using the same process parameters. As illustrated in Figure 2, a similar virus binding and elution trend was observed for both PCV1 and MVM, where MVM level increased with higher conductivity. Unlike PCV1, MVM was undetectable in the first six elution fractions until the NaCl gradient exceeded approximately 17 mS/cm (Figure 2b). MVM LRV from flow through and wash pools was determined to be $\geq$4.93 $\log_{10}$ by the QPCR assay (Table 3).

To further investigate if the removal of PCV1 and MVM virus particles by QSFF correlates with reduction of infectivity of the respective viruses, the samples collected including the spiked load, flow-through pool, wash pool, and elution fractions, were analyzed using infectivity assays. There was

a good correlation between the QPCR data and infectivity for PCV1. There was no detectable PCV1 infectivity in the flow-through pool, wash pool, and the first six elution fractions while a low level of PCV1 QPCR signal was constantly detected across all these pools and fractions, (Figure 2a and Table 2), which can be explained by lower sensitivity of the infectivity assay. There was higher level of infectivity from elution fraction 7 to fraction 13 when elution salt concentration increased (Table 2). The infectivity in the last elution fraction 14 dropped below the assay detection limit (Table 2). It is possible that fractions 11, 12, and 13 represented the peak of PCV1 elution as indicated by the QPCR analysis and the infectivity measurements, resulting in fraction 14 having a titer lower than previous fractions and just below the LOD. There was no infectivity detected in the

Table 3. MVM Distribution and Removal During QSFF Chromatography

Sample	Cond. (mS/cm)	Volume (mL)	DNA (log$_{10}$ GE/mL)	Total DNA (log$_{10}$)	Infectivity (log$_{10}$ TCID$_{50}$/mL)	Total infectivity (log$_{10}$ TCID$_{50}$)
Load	4.2	50.7	7.71	9.42	5.23	6.94
Flow through 1	4.2	25.0	≤2.64	≤4.49*		
Flow through 2	4.2	25.0	≤2.64		≤−0.12*,†	≤1.73*
Wash	4.2	20.5	≤2.64			
Elution Fr. 1	4.5	10.3	≤2.64	≤3.67	NA	NA
Elution Fr. 2	6.5	10.3	≤2.64	≤3.67	NA	NA
Elution Fr. 3	8.6	10.3	≤2.64	≤3.67	NA	NA
Elution Fr. 4	10.6	10.3	≤2.64	≤3.67	NA	NA
Elution Fr. 5	12.6	10.3	≤2.64	≤3.67	≤−0.12	≤0.89
Elution Fr. 6	14.6	10.3	≤2.64	≤3.67	≤−0.12	≤0.89
Elution Fr. 7	16.6	10.3	2.64	3.67	≤−0.12	≤0.89
Elution Fr. 8	18.5	10.3	3.83	4.84	1.12	2.13
Elution Fr. 9	20.4	10.3	5.54	6.55	2.17	3.18
Elution Fr. 10	22.2	10.3	6.66	7.67	NA	NA
Elution Fr. 11	24.1	10.3	7.06	8.08	NA	NA
Elution Fr. 12	26.0	10.3	7.23	8.24	NA	NA
Elution Fr. 13	27.8	13.4	7.76	8.89	NA	NA
Elution Fr. 14	28.5					
LRV‡				≥4.93		≥5.21

*Value determined from combined fractions Flow Through 1, Flow Through 2 and Wash.

†Volumes of 2.5mL Flow through 1, 2.5mL Flow through 2, and 2.7 Wash fraction were combined and applied to infectivity assay.

‡Logarithmic (log$_{10}$) reduction factor calculated from total virus loads in Load-fraction and combined fractions "Flow through 1, Flow through 2, and Wash".

final sanitization fraction presumably due to inactivation by 0.5 N NaOH sanitization buffer.

Like PCV1, the MVM infectivity profile was consistent with the QPCR profile. There was no detectable MVM infectivity and QPCR signal in the flow-through and wash pool (Figure 2b and Table 3). Complete MVM removal from the flow through and wash pool with a LRV of ≥5.21 was determined using the MVM infectivity assay. For sampling during the NaCl gradient elution, fractions 5, 6, 7, 8, and 9 were investigated for infectivity. The QPCR assay was able to detect MVM elution in fraction 7, one fraction earlier than the fraction where MVM infectivity was first detected. This is presumably due to the higher sensitivity of MVM QPCR assay than the infectivity assay used.[31] Fractions 10 to 14 were not assayed for MVM infectivity using NB324K indicator cells. However, MVM infectivity was demonstrated in these fractions using another infectivity assay with A9 indicator cells (data not shown).

The QSFF product flow-through process has been shown to be capable of effectively removing many viruses with a wide range of biochemical and biophysical properties, including the small parvoviruses, for a variety of different mAb purification procedures.[19] In this study, we have used PCV1 as a model virus to investigate removal of PCV by the same QSFF procedure. The data indicated a significant degree of PCV1 viral particle removal and a LRV of 4.12 log$_{10}$ was achieved as measures by QPCR. The observation that high salt concentration disrupts most binding of PCV1 to the QSFF resin supports the idea that an electrostatic mechanism is primarily responsible for its clearance, the same mechanism identified for removal of several other viruses including MVM. However, a relatively weaker PCV1 binding to QSFF was observed compared to MVM binding by the resin. A theoretical explanation for this weaker interaction between PCV1 and QSFF resin could be that the smaller circovirus particles have less negative charges on their surface than the larger parvovirus particles. However, this comparison is currently not feasible because the charge distribution and the *p*I of PCV are unknown. Fur-

ther elucidation of the mechanism of action of PCV removal by QSFF such as determination of the *p*I of PCV will be of interest.

The primary aim of this study was to compare side-by-side removal of PCV1 and MVM by QSFF chromatography without any potential effect of additional proteins. Therefore, the feedstocks used in this study did not contain monoclonal antibody. However, impact from the monoclonal antibodies on virus reduction has not been observed thus far under the conditions tested when antibodies flow through. For example, viral removal of over 20 Roche monoclonal antibodies was validated with X-MuLV and MVM. Similar high levels of viral removal were observed (data not shown), consistent with industry observation.[19] In addition, two antibodies with *p*I of 6.8 and 8.8, with or without resin binding, appeared to have no effect on virus binding to the resin.[34] In theory, high levels of impurities could compete with virus for binding sites on QSFF resin thus affecting virus reduction. Further elucidation of the robustness of PCV removal over a range of process parameters and in the presence of intermediates from monoclonal antibody purification will be of great interest.

Thus far, there is no indication of productive PCV infection in humans by contaminated, orally administered rotavirus vaccines.[35] However PCV has been shown to productively infect certain human cells *in vitro*.[36] PCV DNA has also frequently been detected in human stools and pork products.[37] While the health risk from oral consumption of PCV seems extremely low, human PCV infection still remains of theoretical concern from parenteral drugs if contamination happens. Regulations with regard to PCV contamination are limited and not well established, for example, regulation US 9CFR113.53 does not consider PCV. However, the US FDA now lists circoviruses in their guidance on testing of cell substrates and biological materials used in the production of live virus vaccines[38] and recent European Monograph 5.2.3 for cell substrates for vaccines[39] is changing to consider circoviruses. In addition, EMA guidance on porcine trypsin is in preparation.[40]

Conclusions

We have shown that a chromatographic purification step such a QSFF chromatography can play an important role for virus clearance at down-stream processing of recombinant proteins from cell culture. The extremely small circoviruses such as PCV are not effectively removed by the widely used virus filters and QSFF chromatography can be used as a complimentary effective virus clearance step for such viruses. This study demonstrates a promising solution to address concerns on circovirus contamination. The virus clearance mechanism of QSFF chromatography is known to be charge based, which is similar to that of other impurity removal by QSFF resin. Due to their slightly acidic pI, a wide range of viruses bind to the resin where most mAbs with higher pI values flow through during the regular QSFF process at neutral or slightly basic pH. In our study, PCV1 could be removed in a similar way as MVM by binding to the resin. It remains intriguing though to further investigate if presumably different pI or surface charge distribution of PCV1 caused the somewhat weaker binding strength of PCV1 to QSFF resin than that of MVM.

Acknowledgments

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