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Use of tangential flow filtration for improving detection of viral adventitious agents in cell substrates

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ABSTRACT

In this study, we assessed the feasibility of tangential flow filtration (TFF) for primary concentration of viral adventitious agents (AAs) from large volumes of cell substrate-derived samples, such as cell-free Chinese hamster ovary (CHO) culture supernatants (500 mL) and CHO cell lysates (50 mL), prior to virus detection in them by nucleic acid-based methods (i.e., qPCR and massively parallel sequencing (MPS). The study was conducted using the samples spiked with four model DNA viruses (bovine herpesvirus type 4, human adenovirus type 5, simian polyomavirus SV-40, and bovine parvovirus). The results showed that the combined TFF/MPS approach enables reliable detection of as low as 1000 genome equivalents (GE) of each of the four viruses spiked into the cell substrate samples. The final achieved sensitivities of 2 GE/mL for cell culture supernatant and 20 GE/mL for cell lysate make this approach more sensitive than virus-specific PCR and qPCR assays. The study results allowed us to propose that TFF might be useful and valuable method for simple and rapid concentration of potential AAs in cell substrate samples prior to AAs detection by conventional *in vivo*, *in vitro*, or molecular methods.

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1. Introduction

Manufacture of viral vaccines and other cell-derived biopharmaceuticals is a complex technological process, which includes the use of different biological materials (e.g., a variety of cell substrates such as embryonated chicken eggs, primary cell cultures, and continuous cell lines, raw materials and media additives of different host origins, and virus seeds). The use of such materials and the complexity of manufacture of cell-derived biopharmaceutical products make the entire process potentially vulnerable to accidental contamination with adventitious agents that may be inadvertently introduced with the materials used or from environmental sources [1–4].

It is important to point out that while the detection of most bacterial contaminants is facilitated by the use of well-developed microbiological tests, the detection of viral AAs represents a considerable methodological and technical challenge and necessitates using a large battery of *in vivo*, *in vitro*, and molecular assays [5]. However, despite all rigorous measures undertaken in the biotech industry to prevent potential contamination, the rare events of contamination of cell substrates, as well as cell-derived biopharmaceuticals, with viral AAs continue to occur [6]. As it has been demonstrated, in some cases the contamination was caused by previously unknown viruses or viruses not previously considered as potential adventitious agents of cell substrates [1–3,7–9].

Increasing demand of a biotech industry in new mammalian, insect, and other origins of cell substrates for manufacture of innovative biopharmaceuticals and new vaccines requires development and implementation of new rapid, reliable, and highly sensitive biosafety assays able to detect a broad spectrum of viral AAs including both known and yet unknown species. Therefore, the discussion of advantages and perspectives of implementation of advanced molecular techniques, such as e.g., MPS, able to benefit the biosafety testing of cell-derived biological products, was a key topic of several recently held scientific and regulatory meetings [10–13].

The presence in tested samples of biological matrices such as a host DNA and other cellular and media components able to interfere with the efficient detection and genetic analysis of AAs by molecular methods may significantly reduce the method's sensitivity due to the restricted quantities (amount, volume) of a sample that can be used for testing [10,14]. Therefore, development of

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novel sample preparation methods enabling sufficient and selective concentration of AAs from large volumes of test articles can lead to more efficient detection of low AAs contamination levels and result in broader application of molecular methods for biosafety testing in cell substrates and cell-derived pharmaceuticals [14].

By now, a variety of different concentration methods have been developed and some of them were successfully applied in metagenomics studies for concentration of microbes from large volumes of environmental samples. These methods employ different technical approaches including flocculation, precipitation, ultracentrifugation, filtration, absorption-elution, and ion-exchange chromatography [15–20].

The goal of our current study was to assess the feasibility of tangential flow filtration technique (TFF) for concentration of viral adventitious agents from relatively large volumes (up to 500 mL) of cell substrate samples, such as cell-free culture supernatants and cell lysates spiked with different amounts of four selected DNA viruses, followed by virus detection using quantitative qPCR and MPS. The concentration was performed using specially pretreated hollow-fiber filters [21] with 100 kDa molecular weight cut-off (MWCO) and membrane pore sizes able to retain a broad range of known DNA and RNA viruses including parvoviruses and circoviruses, which have the smallest virion sizes (approximately 17–26 nm) [22]. The main advantage of TFF over conventional dead-end filtration methods is that the solution stream during TFF flows along the surface of the TFF membrane preventing the sedimentation of large molecules on the filter surface and reducing filter fouling [23]. Due to the ability to process larger volumes of liquid samples in comparison with the conventional filtration methods, TFF is widely used in metagenomics studies of environmental specimens [21], for removal of potential adventitious agents from different biopharmaceutical products [24], and concentration of viral antigens and viral vectors from the viral harvest stage during manufacturing [23,25–28].

2. Materials and methods

2.1. Cell cultures and viruses

All cell cultures were grown at 37 °C under humidified 5% CO_2. Chinese hamster ovary cells (CHO-K1 cells, ATCC CCL61) were propagated either in F12 media (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) or 10% CDM-HD serum replacement (FiberCell System Inc., Frederick, MD) in the presence of penicillin (100 units/ml), streptomycin (100 μg/ml) (Life Technologies, Carlsbad, CA). HEK-293 (ATCC CRL-1573), MDBK (ATCC CCL-22), BT (ATCC CRL-1390) and CV-1 (ATCC CCL-70) cells were used for propagation and preparation of four DNA virus stocks selected to assess the feasibility of tangential flow ultrafiltration for concentrating adventitious viruses from cell substrates (Table 1). These four cell cultures were grown in DMEM or MEM media supplemented with 10% FBS or horse serum (Table 1). After infection with viruses, the cells were incubated in the presence of media containing 2% FBS or horse serum. For a large-scale preparation of selected viruses, several 175-cm^2 flasks (Corning Inc, Corning,NY) containing 60–70% confluent monolayers of permissive cells were infected with each virus at multiplicity of infection (MOI) ≥1 (the number of cells per flask was estimated using the Corning Inc. cell confluence chart (http://csmedia2.corning.com/LifeSciences/media/pdf/an_surface_areas_reco_med_vol_for_cc_vessels.pdf) and incubated until the 60–70% cytopathic effect (CPE) was observed (usually 2 or 3 days post-infection). The cells were subjected to a triple freeze/thaw procedure and cell debris was removed by centrifugation at 2000 g for 10 min and supernatant was additionally clarified by filtration through 0.2-μm Filter Units (Millipore Corporation, Billerica, MA). All viruses were purified using two separate ultracentrifugations through a 20%–40% iodixanol gradient (Axis-Shield Inc., Oslo, Norway) at 22,000 g for 3 h at +4 °C. The banded virus was identified by qPCR and infectivity testing of gradient fractions. The residual cellular DNA and non-encapsidated viral DNA were removed by additional treatment of viral stocks with 0.5 U/μL of bovine DNase-I (Sigma–Aldrich Co., St. Louis, MO) for 1 h at +37 °C as described previously [29]. The final viral stocks were used for isolation of viral genomic DNA by means of a High Pure Viral Nucleic Acid Large Volume Purification Kit (Roche Inc., Indianapolis, IN). The purified viral genomic DNAs were quantified with Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies, Carlsbad, CA) and used as virus-specific DNA standards for the qPCR-based virus GE number assessment. The single stranded parvovirus genomic DNA was quantified using a Qubit™ ssDNA Assay Kit (Life Technologies, Carlsbad, CA).

Virus-specific qPCR primers used for the GE enumeration and shown in Table 2 were designed with OligoPerfect Designer (Life Technologies, Carlsbad, CA). qPCR assays were performed on a 7900 HT real-time PCR (Life Technologies, Carlsbad, CA) using universal amplification conditions recommended by the manufacturer's protocol for the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA). Briefly, a standard qPCR mixture contained 12.5 μl QuantiTect SYBR Green PCR Master Mix, 12 pmoles of each forward and reverse primers, 10 μl DNA template and DNase-free water up to 25 μl. The qPCR was performed using the following conditions: 1st step – DNA denaturation and Taq polymerase activation at +95 °C for 15 min; 2nd step – 45 cycles of denaturation at +94 °C for 15 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s; 3rd step – final elongation at 72 °C for 5 min; and 4th step – dissociation curve analyses of synthesized PCR products. The number of viral genomic equivalents in each sample was calculated based on a comparison of sample Ct values with that obtained for serial dilutions of the standard virus-specific DNA. All qPCRs, including standard dilutions, were performed in duplicates. The qPCR data were analyzed using the 7900 HT SDS 2.3 software. The GE numbers were determined as an average from two independent PCR experiments.

The median tissue culture infective doses ($TCID_{50}$) of prepared reference virus stocks were determined in replicate for serial dilutions of the virus samples [30] and calculated according to the Spearman-Karber method [31].

2.2. Virus concentration assessment by epifluorescence microscopy

An independent counting of virus-like particles was performed using EPF microscopy of viral samples after staining of viral nucleic

Table 1
Virus-specific qPCR primers used for GE quantification of the selected reference viruses.

Virus	GenBank accession number	Forward primer	Reverse primer
Human adenovirus type 5	AC_000008	5′GACATGACTTTCGTTCGATCCCATGGA 3′	5′CCGGCTGAGAAGGGTGTGCGCAGGTA 3′
Bovine parvovirus	M14363	5′CAGACCTGCTAAGACTGAGATACTCC 3′	5′CCAGGAGTGTCTTTCTGTTGACTC 3′
Bovine herpesvirus type 4	JN133502	5′GGGTTGTTGAGTGAGACTGTAAGG 3′	5′CTCTATGTCACGTGTCCAACCTAC3′
Simian polyomavirus SV-40	AY271817	5′TGGAGGAGTAGAATGTTGAGAGAG 3′	5′AACCTATGGAACTGATGAATGG 3′