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Vesivirus 2117: Cell line infectivity range and effectiveness of amplification of a potential adventitious agent in cell culture used for biological production

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ABSTRACT

Vesivirus 2117 (VV-2117) has been recently described as a contaminant of cell culture operations in several biologics manufacturing facilities. VV-2117 has been poorly studied and little information exists about its biology, pathogenicity and infectivity range in cell culture settings. In this study we evaluated the potential *in vitro* viral infectivity of VV-2117 using a range of established mammalian cell lines from various species, the effectiveness of virus amplification in CHO-K1 cells at differing infection levels, and the relative sensitivity of two test methods (cytopathic effect [CPE] and polymerase chain reaction [PCR]) to detect infection and viral amplification. Of eight cell culture systems studied, two originating from hamster (CHO-K1 and BHK-21) and one from canine (MDCK) were positive for CPE and also showed a marked increase of viral RNA in a reverse transcriptase quantitative PCR (RT-qPCR) test. CHO-K1 cell cultures inoculated at 10, 1 and 0.1 genome copies per cell (gc/cell) showed both CPE and amplification of VV-2117 RNA, indicating that infection had occurred in these cultures. CHO-K1 cultures inoculated at 0.01, 0.001, 0.0001 and 0.00001 gc per cell showed neither CPE nor VV-2117, indicating that infection had not occurred. Therefore, the minimum dose necessary for infection of CHO-K1 cells was approximately 0.1 genome copies per cell. At any infection level where VV-2117 amplification was observed by RT-qPCR, the cultures also showed CPE. There was no low-level infection that could be detected by RT-qPCR without developing signs of CPE. However, the RT-qPCR assay appeared more sensitive in that it detected VV-2117 infection earlier than the onset of observable CPE.
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1. Introduction

The use of continuous cell lines in the manufacture of biological products such as vaccines, recombinant proteins and monoclonal antibodies is associated with a concomitant risk of cell substrate contamination with endogenous retroviruses, latent viruses, or new and emerging adventitious agents. A number of regulatory guidance documents have been enacted governing product safety from adventitious agents [1–10]; thus, the risk of adventitious agent contamination is an important focus of regulatory attention during biological product development and production.

Vesivirus 2117, a member of the *Caliciviridae* family, has been recently described [11] as a contaminant of cell culture operations in a biologics manufacturing facility. Subsequently, genotypes of the same virus have been incriminated in three more commercial bio-production contaminations of cell culture operations [12,13].

Caliciviridae is a family of small round viruses that are ubiquitous in nature, both in marine mammals and in certain terrestrial animals. Virions are nonenveloped, 27–40 nm in diameter, and have icosahedral symmetry. Some virions have a characteristic appearance with 32 cup-shaped depressions on their surface, whereas enteric calicivirus virions lack the characteristic surface decorations and have a fuzzy appearance [14]. The genome consists of a single molecule of a linear, positive sense, ss-RNA, 7.4–8.45 kbp in size. Genomic RNA acts as the mRNA and is infectious. The 5′-end of the genome has a genome-linked protein (VPg), whereas the 3′-

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terminus is polyadenylated. The RNA genomes of the noroviruses and vesiviruses are organized into three major open reading frames (ORF1, -2, and -3). The ORF1 encodes a large polyprotein that is proteolytically processed into the mature nonstructural proteins. The ORF2 encodes the major capsid protein, VP1, and ORF3 encodes a minor structural protein, VP2 [14].

The family comprises 6 genera, *Vesivirus*, *Lagovirus*, *Norovirus*, *Sapovirus*, *Recovirus* and *Beco*/*Nebovirus* [15,16].

Although many animal caliciviruses have been well published and their natural reservoirs and pathogenicity are known, VV-2117, being a relatively new agent, has been poorly studied. Little or no information exists about its natural reservoirs, animal host range, maintenance in the nature, role in human and animal health, virus infectivity range in cell culture settings, and virus resistance to physical and chemical factors.

The purpose of this study was to evaluate (a) *in vitro* viral infectivity using a range of established mammalian cell lines from various species, (b) effectiveness of virus amplification in CHO-K1 cells at various infectious doses of virus (expressed as gc/cell), (c) respective sensitivities of two detection methods, cytopathic effect (CPE) in cell culture and detection of viral nucleic acid by RT-qPCR assay, and (d) determine whether a low-level infection could be established that showed amplification of virus by RT-qPCR assay without development of CPE.

2. Methods

2.1. Evaluation of different cell lines for their ability to support infection and amplification of vesivirus 2117

2.1.1. Cells

Eight mammalian cell lines derived from seven different species were studied (Table 1). These cell types are frequently used in commercial manufacturing of bio-therapeutics (biologics) and in diagnostic laboratories for routine virus detection.

2.1.2. Virus

The virus used in the study was VV-2117, strain *Calicivirus Allston 2008/US*, which was originally isolated from a contaminated bio-production reactor [12,13]. The virus was laboratory adapted after four passages in CHO cell culture. This strain shares 89% nucleic acid sequence homology with the original strain described (unpublished data).

Although the virus was originally isolated from a contaminated CHO cell culture bioreactor, it could not always be detected in the *in vitro* cell culture assay, even when using CHO-K1 cells. It produced inconsistent CPE and hemadsorption. When it was detected, the onset of CPE varied from day 5 to day 11 in spite of very high virus concentration as measured by PCR specific for VV-2117 sequences. Frequently, the CPE would disappear after a few days and infected cultures would appear healthy. Due to these issues, the infectivity titer was difficult to assess with reliability. A ratio of 10^4–10^5 $TCID_{50}$/mL to more than 10^9 gc/mL was determined during the early virus isolation and investigation phase (unpublished data).

Because of the inconsistent CPE and the inability to detect infection reliably, the genome copy number was used for virus quantification in this study as more precise method rather than a classical tissue culture infectivity technique. The viral titer was determined to be 6.78×10^9 gc/mL. The virus was stored frozen at −70 °C, and for this study it was first propagated in CHO cells to make a fresh virus stock.

Table 1
Cell lines used in the study.

#	Cell line	Species of origin	Source of cells
1	CHO-K1	Hamster	ATCC CCL-61
2	VERO	Monkey	ATCC CCL-81
3	MRC-5	Human	ATCC CCL-171
4	NIH-3T3	Mouse	ATCC CRL-1658
5	MDCK	Canine	ATCC CCL-34
6	BHK-21	Hamster	ATCC CCL-10
7	MDBK	Bovine	ATCC CCL-22
8	ST	Porcine	ATCC CRL-1746

2.1.3. Virus inoculation and cell culture observations

Each cell line was cultured in 25 cm^2 culture flasks. Cultures were subconfluent at the time of inoculation. One flask of each cell type was uninfected and served as negative control. An amount of vesivirus inoculated into one flask of each cell type was the same, 50–60 gc/cell, based on the prior studies of virus amplification in CHO-K1 cells. CHO-K1 cells, also inoculated with virus at 50–60 gc/cell, served as the positive virus amplification control. Inoculated cultures were incubated for 60 ± 10 min at 37 °C and then refed with medium appropriate for the cell line containing 5% gamma irradiated FBS.

All flasks were then incubated at 37 °C in a humidified 5% CO_2 atmosphere and observed for CPE at least twice per week throughout the culture period. After one week, all flasks were frozen at −70 °C or below to lyse the cells. The resulting lysate was centrifuged to remove solid material. Supernatant samples were collected for analysis for vesivirus by RT-qPCR, and also for subpassage onto subconfluent fresh cultures of the same cell type. Samples for RT-qPCR were stored at −70 °C until testing. At the end of a second week, flasks were frozen as before and supernatant samples were collected for subpassing and analysis as before. At the end of a third week, flasks were frozen and supernatant samples were collected and stored at −70 °C for further analysis. The study was terminated at the end of third week. Cultures showing CPE were harvested when extensive CPE was observed and no additional passage was performed.

2.1.4. RT-qPCR assay

Vesivirus RNA was extracted from 200 μL of culture supernatant using a GeneJet Viral RNA and DNA Purification kit from ThermoFisher Scientific and was eluted with 100 μL of elution buffer. The real-time reverse transcription-qPCR (RT-qPCR) method for the detection of vesivirus was developed and optimized at Eurofins Lancaster Laboratories. The primers and probe were designed based on published sequences from VV-2117 genome (GenBank accession number AY343325.2). The PCR amplicon is a 60 bp fragment of the non-structural polyprotein gene (GenBank accession number AAQ24845.2) within the genome of VV-2117. The primers and probes detect all identified strains of VV-2117.

Synthetic RNA encoding the RT-qPCR target sequence was serially diluted to prepare the standard curve. The standard curve consisted of eight dilutions in concentrations ranging from 2×10^3 to 1×10^{10} genome copies per mL (gc/mL). The supernatant of an uninfected cell culture was used as a negative control for the assay. The supernatant of an uninfected cell culture spiked with approximately 2.8×10^6 genome copies of VV-2117 was used as a positive control for the assay.

All RT-qPCR runs were performed in a 50-μL reaction volume containing 20 μL of RNA extract, 25 μL of RT-PCR master mix (RNA to CT kit, ThermoFisher), 1.25 μL RT Enzyme Mix (RNA to CT kit, ThermoFisher), forward and reverse PCR primers at a final concentration of 900 nM, and PCR probe at a final concentration of 250 nM. The thermal cycling conditions were as follows: reverse transcription (RT) at 48 °C for 30 min; reverse transcriptase

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