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A rapid *Orthopoxvirus* purification protocol suitable for high-containment laboratories

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A B S T R A C T

Virus purification in a high-containment setting provides unique challenges due to barrier precautions and operational safety approaches that are not necessary in lower biosafety level (BSL) 2 environments. The need for high risk group pathogen diagnostic assay development, anti-viral research, pathogenesis and vaccine efficacy research necessitates work in BSL-3 and BSL-4 labs with infectious agents. When this work is performed in accordance with BSL-4 practices, modifications are often required in standard protocols. Classical virus purification techniques are difficult to execute in a BSL-3 or BSL-4 laboratory because of the work practices used in these environments. Orthopoxviruses are a family of viruses that, in some cases, requires work in a high-containment laboratory and due to size do not lend themselves to simpler purification methods. Current CDC purification techniques of orthopoxviruses uses 1,1,2-trichlorotrifluoroethane, commonly known as Genetron®. Genetron® is a chlorofluorocarbon (CFC) that has been shown to be detrimental to the ozone and has been phased out and the limited amount of product makes it no longer a feasible option for poxvirus purification purposes. Here we demonstrate a new *Orthopoxvirus* purification method that is suitable for high-containment laboratories and produces virus that is not only comparable to previous purification methods, but improves on purity and yield.

1. Introduction

Virus purification in a high-containment setting provides unique challenges due to barrier precautions and operational safety approaches that are not necessary in lower biosafety level (BSL) 2 environments. The need for high risk group pathogen diagnostic assay development, anti-viral research, pathogenesis and vaccine efficacy research necessitates work in BSL-4 labs with infectious agents. When this work is performed in accordance with BSL-4 practices, modifications are often required in standard protocols. Classical virus purification techniques are difficult to execute in a BSL-3 or BSL-4 laboratory because of the work practices used in these environments. The minimal use of sharp objects in the BSL-4 labs restricts the use of glass items, such as a dounce homogenizer, or needles as would be needed for certain gradient extractions. The visibility and dexterity in either a full-body, air-supplied, positive pressure suit, or a class III biosafety cabinet also make procedures like visual identification of gradient layers containing virions in gradient centrifugations more difficult. Under some instances, methods employed to purify small virus particles by filtration of culture media might not be suitable for larger pathogens (Berting et al., 2005; Ver et al., 1968). Hence, there is a need for pathogen specific protocols to produce purified preparation of viruses. In this study, we have addressed an alternate protocol for simple and efficient purification of *Variola virus* (VARV), a BSL-4 pathogen.

VARV, the causative agent of smallpox, belongs to the *Orthopoxvirus* genus of the family *Poxviridae*. The poxviruses includes large double stranded DNA (dsDNA) viruses, approximately 360 × 270 × 250 nm, with a wide host range of infectivity, from insects to mammals. One of the unique features of poxviruses is the ability to replicate and assemble into the fully infectious form in the cytoplasm of infected cells (Cyrklaff et al., 2005; Moss, 2012). The majority of mature infectious virions (MVs) accumulate in the cytoplasm with only a small portion, <1%, secreted in the form of extracellular virus (Payne, 1979; Payne, 1986). Hence, the purification of poxviruses are primarily of the MV form from cell lysates.

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Infected cells are sheared using dounce homogenizers suspended in hypotonic lysis buffer or through the use of repeated freeze/thaw cycles, followed by separation using density gradient centrifugations (Broder and Earl, 1999; Huang and Chang, 2012; Kremer et al., 2012). Although, this method is widely employed in BSL-2 laboratories for purification of most orthopoxviruses such as *Vaccinia virus* (VACV) and *Cowpox virus* (CPXV), safety precautions prevent use of this procedure in high-containment laboratories, while working with *Monkeypox virus* (MPXV) (BSL-2 with BSL-3 practices and BSL-3 if working with infected animals) or VARV (BSL-4) (Canada, 2011; Services et al., 2009).

Current CDC purification techniques of orthopoxviruses uses 1,1,2-trichlorotrifluoroethane, commonly known as Genetron®. Genetron®, aids in the disassociation of virus particles from cellular membranes and has been used in poxvirus purification, hepatitis A vaccine investigation studies, purification of previously unknown viruses such as fowl poxviruses and epizootic hemorrhagic disease virus among other virus purifications (Al Falluji et al., 1979; Bogaerts and Durville-van der, 1972; Epstein, 1958; Heinricy et al., 1987; Kontor and Welch, 1976; Welch, 1971). Genetron® is a chlorofluorocarbon (CFC) that has been shown to be detrimental to the ozone and the Montreal protocol called for the phase out of Genetron® use by 2010. Though there are exceptions in the United States for the use of Genetron®, the limited amount of product makes it no longer a feasible option for poxvirus purification purposes. Due to the need for purified MPXV and VARV in diagnostic assay and animal model development, a new purification protocol lacking Genetron® and employing a deoxyribonuclease (Benzonase®) was utilized for purification. Initial experiments were performed and optimized using VACV in a BSL-2 laboratory and followed up with VARV and MPXV purification in BSL-4 or BSL-3 labs respectively. In this study, we present the efficacy of the new Benzonase® protocol compared to Genetron® purification for high-containment laboratories.

2. Materials and methods

2.1. Viruses

MPXV strain USA 2003_44 (accession #DQ011153),VACV strain Wyeth, and VARV strain Bangladesh 1975 (accession #L22579) were used in this study. VARV is a Tier-1 select agent and subject to the select agent regulations 42 CFR Part 73. Per Biosafety in Microbiological and Biomedical Laboratories recommendations, all of the staff who participated in this work were vaccinated (U.S.D.o.H.a.H Services, 2009). MPXV and VACV were grown at 37 °C and VARV was grown at 35.5 °C. Initial experiments with MPXV and VACV were conducted in a BSL-2 laboratory using BSL-3 precautions and experiments with live VARV were performed in a BSL-4 laboratory. BSC-40 cells (African green monkey kidney cells) from eight T150 flasks, infected at a multiplicity of infection (MOI) of 0.1, were harvested at optimal observable cytopathic effects (CPE), approximately 48 h post-inoculation (hpi).

2.2. Virus purification

2.2.1. Benzonase®-positive and Benzonase®-negative purifications

The infected cells were scraped from the flasks and combined, then centrifuged at 15,300 × *g* in a Beckman J14 rotor for 1 h. The pellet was resuspended in 60 ml total volume of 1 mM Tris–HCL (pH 9.0) (sample 1) and divided in half placing 30 ml into each of two, 50 ml conical tube. Both suspensions were sonicated three times in a cup horn sonicator (Branson Digital Sonifier® 450) containing water maintained at 4 °C with ice for 1 min at 160 W, vortexing and placing the tubes on ice for 20 s between each sonication and replacing the ice water between sonications. Five μl of Benzonase® (≥250 units/uL; Sigma-Aldrich #E1014-25KU CAS#9025-65-4), St. Louis, MO) was added to one sample (sample B+) and incubated at room temperature for 30 min while the other sample was left on ice (B-). Samples were centrifuged for 10 min at 1174 × *g* in a Sorval ST 16R and the resulting supernatants (approximately 25–30 ml) were laid over a 5 ml 36% sucrose cushion in a 50 ml polycarbonate Oak Ridge centrifuge tube (Beckman #3118-0050, Brea, CA) and centrifuged for 80 min at 22,600 × *g* in a Beckman JS13.1 rotor. The supernatants were removed and each pellet resuspended in 5 ml 1 mM Tris–HCL (pH 9.0) and sonicated as described above. One milliliter was removed for analysis. The remaining 4 ml of suspension was placed over a 1 ml 36% sucrose (w/v in 1 mM Tris–HCl pH 9.0 buffer) cushion in a 5 ml sterile polyallomer centrifuge tube (Beckman #326819) and centrifuged for 80 min at 30,000 × *g* in a Beckman SW55Ti swinging bucket rotor. Again the supernatant was removed and the final pellet was resuspended in a total volume of 1 ml 1 mM Tris–HCL (pH 9.0) and sonicated as described previously. A diagram of the experimental outline demonstrates the steps (Supplementary Fig. 1). Samples were taken at each step for analysis.

2.2.2. Genetron® purification

The Genetron® purification was modified from the original protocol (Epstein, 1958) and performed as described previously (Hughes et al., 2014). The infected cells were scraped from the flasks and combined, then centrifuged at 15,300 × *g* in a Beckman J14 rotor for 1 h at 4 °C. The pellet was resuspended in 20 ml total volume of Tris–HCL (pH 9.0). The suspension was sonicated three times at 160 W for 1 min, vortexed and placed on ice for 20 s between each cycle. The ice water was replaced between sonications. Samples were then centrifuged at 4 °C for 10 min at 1174 × *g* in a Sorval ST 16R and the supernatants were collected, combined, and placed on ice. The pellets were resuspended in 10 ml total volume of 1 mM Tris–HCL pH 9.0. Samples were sonicated as described above. The suspensions were centrifuged at 1174 × *g* in a Sorval ST 16R for 10 min at 4 °C, supernatants were collected and combined with preparations from the previous spin for a total volume of 30 ml. This volume was then divided into two 50 ml conical tubes containing 15 ml each. Twenty ml of Genetron® was added to each tube, vortexed twice for 30 s, to achieve a homogeneous viscous mixture, and then centrifuged at 1174 × *g* for 5 min at 4 °C. The upper aqueous phase was collected and placed into a new tube. An equivalent volume of Genetron® was added to each tube and the samples were centrifuged again at 1174 × *g* for 5 min at 4 °C. The resulting supernatants (approximately 25–30 ml) were overlaid onto a 5 ml 36% sucrose cushion in a 50 ml polycarbonate Oak Ridge centrifuge tube (Beckman #3118-0050) and centrifuged for 80 min at 22,600 × *g* in a Beckman JS13.1 rotor. The supernatants were discarded and pellet was resuspended in 5 ml of Tris–HCL and sonicated three times. The suspensions (4 ml) were placed over a 1 ml 36% sucrose cushion in a 5 ml sterile polyallomer centrifuge tube (Beckman #326819) and centrifuged for 80 min at 30,000 × *g* in a Beckman SW55Ti swinging bucket rotor. The supernatants were discarded and the final pellets were resuspended in a total volume of 1 ml 1 mM Tris–HCL (pH 9.0) and sonicated as described previously. An aliquot of all samples were taken at each step for analysis.

2.3. Real-time PCR

2.3.1. Generic orthopoxvirus PCR

Orthopoxvirus DNA was extracted using the QIAamp DNA Blood Mini Kit per manufacturer's instructions (Qiagen, Valencia, CA). The *Orthopoxvirus* generic PCR was performed as previously described (Reynolds et al., 2010). In short, primers and probes targeting the