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Production, Purification, and Capsid Stability of Rhinovirus C Types

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Abstract

The Rhinovirus C (RV-C) were discovered in 2006 and these agents are an important cause of respiratory morbidity. Little is known about their biology. RV-C15 (C15) can be produced by transfection of recombinant viral RNA into cells and subsequent purification over a 30% sucrose cushion, even though yields and infectivity of other RV-C genotypes with this protocol are low. The goal of this study was to determine whether poor RV-C yields were due to capsid instability, and moreover, to develop a robust protocol suitable for the purification of many RV-C types. Capsid stability assays indicated that virions of RV-C41 (refractory to purification) have similar tolerance for osmotic and temperature stress as RV-A16 (purified readily), although C41 is more sensitive to low pH. Modification to the purification protocol by removing detergent increased the yield of RV-C. Addition of nonfat dry milk to the sucrose cushion increased the virus yield but sacrificed purity of the viral suspension. Analysis of virus distribution following centrifugation indicated that the majority of detectable viral RNA (vRNA) was found in pellets refractory to resuspension. Reduction of the centrifugal force with commiserate increase in spin-time improved the recovery of RV-C for both C41 and C2. Transfection of primary lung fibroblasts (WisL cells) followed by the modified purification protocol further improved yields of infectious C41 and C2. Described herein is a higher-yield purification protocol suitable for RV-C types refractory to the standard purification procedure. The findings suggest that aggregation-adhesion problems rather than capsid instability influence RV-C yield during purification.

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CONFLICT OF INTEREST

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Keywords

Rhinovirus C; purification; concentration; stability; centrifugation

1. INTRODUCTION

Rhinoviruses (RVs), the agents of most common colds, can contribute to more severe illnesses in susceptible individuals. For example, children, the elderly, the immunosuppressed and those with chronic respiratory conditions, such as asthma or COPD, are more predisposed to lower respiratory illnesses resulting in hospitalization (Brownlee and Turner, 2008; Cox et al., 2013; Gern, 2010; Miller et al., 2007; Peltola et al., 2008). Recent studies have demonstrated that respiratory illness severity is correlated with the type of RV, and the most severe colds and asthma exacerbations are likely caused by isolates in the RV-A and C species (Cox et al., 2013; Lee et al., 2012).

Of the three RV species, the RV-A and B were identified decades ago, while RV-C were only recently discovered (2006) via molecular techniques (Arden et al., 2006; Lamson et al., 2006; Price, 1956). RV-C isolates are unable to grow in standard immortalized cell lines used to culture other species of RV, because these cells lack the required cellular receptor. Sequence analysis and molecular modeling has made it clear the RV-C have large capsid protein alterations that are associated with receptor specificity (Basta et al., 2014). The inability to grow RV-C in standard tissue culture initially prevented a detailed investigation of these isolates, until Bochkov *et al* developed a reverse genetics system for RV-C15 (C15) that produced functional virions after transfection of RNA synthesized *in vitro* into cell monolayers (Bochkov et al., 2011). The resultant C15 material was successfully purified using centrifugation through sucrose cushions and related protocols developed for the A and B viruses (Lee et al., 1995, 1993; Sherry et al., 1986; Wang et al., 1998).

While this protocol worked well for C15, a marked loss in viral yield and infectivity was consistently observed when the protocol was applied to other C genotypes (e.g. C41 and C2) (Nakagome et al., 2014). To date, obtaining high yields of RV-C other than C15 has been problematic because of technical difficulties in virus recovery procedures. Large stocks of purified RV-C are needed for structure determinations, monoclonal antibody production, immunological studies, and other RV-C investigations (Nakagome et al., 2014; Pande et al., 2010; Rossmann, 2013).

These findings suggested the hypothesis that some C types have unstable capsid structures that cause apparent low viral yields following standard purification procedures. To test this hypothesis, experiments were conducted to test stability of RV-C capsids to thermal, osmotic and acid stress. The results did not support this hypothesis, and instead led to the development of a revised purification protocol designed to reduce virion aggregation and improve viral yields. Virions purified in this fashion retain infectivity for primary cultures of differentiated airway epithelial cells.

2. MATERIALS & METHODS

2.1. Viruses

RV-A16, C2, C15, and C41 samples from clinical isolates were cloned into cDNA, linearized, transcribed into infectious RNA (RiboMAX™ Large Scale RNA Production System-T7, Promega, Madison, WI), and then transfected into HeLa or WisL (fetal lung fibroblast) cells (Lipofectamine® 2000 transfection reagent, Life Technologies, Grand Island, NY) as previously described (Bochkov et al., 2011; Lee et al., 1995; Nakagome et al., 2014; Wang et al., 1998).

2.2. Cell Culture

HeLa (ATCC CRL-1958) and WisL cells were grown in monolayers as previously described (Bochkov et al., 2011; Watters and Palmenberg, 2011). All experiments utilized HeLa cells for virus production, unless otherwise specified. Primary human bronchial epithelial cells (HBECs) were extracted from surgical specimens provided by the University of Wisconsin Department of Surgery, Division of Transplantation and grown by the air-liquid interface (ALI) culture method as described previously (Ashraf et al., 2013; Hao et al., 2012). HBEC ALI cultures were inoculated with $\sim 10^6$ units of purified virions (PCR genome equivalents) per well or with BEGM (Lonza) alone for infectivity assay.

2.3. Solutions

Stock solutions of $50\times$ 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer were prepared by adding dry HEPES (Sigma) to a final concentration of 1.25M in DPBS, and adjusting the pH to 7.3-7.4. Sucrose (30% w/v, pH 7.1-7.2) was prepared in DPBS and 2% (v/v) 50X HEPES. Stocks of 0.1M citric acid and 0.2M Na_2HPO_4 were prepared and then mixed in required combinations (Table 1) to attain pH 5.5 – 7.0.

2.4. Rhinovirus C Concentration, Revised Protocol

All steps of this procedure were performed in a laminar flow hood using sterile cell culture material and solutions. 24 hours post transfection of WisL cells, T-75 flasks were removed from incubator and 200 μl of $50\times$ HEPES was added per 10mL of media in each flask. Flasks were incubated at -80°C for 20min, and then at room temperature for 30min or at 37°C for 15min (until cultures were thawed fully). This cycle was repeated twice more. After the third thaw, adherent cellular debris was scraped from the flasks, and cell lysates were transferred to 30mL round-bottom centrifuge tubes (Thermo Scientific Nalgene Oak Ridge, Cole-Parmer, Vernon Hills, IL). Lysates were vortexed and pelleted ($10,000 \times g$, 10min, 4°C). Supernatants were decanted into sterile 50mL conical tubes, and treated with RNase A (100 $\mu\text{g}/\text{mL}$, 37°C , 10min; Qiagen) to remove transfection input RNA. B-mercaptoethanol was then added to a final concentration of 0.2% (v/v). Lysates were mixed briefly, and 10 μl aliquots were saved in 90 μl DPBS for qPCR assay of treated lysate. Sucrose cushions (1mL/tube) were prepared in $14 \times 89\text{mm}$ centrifuge tubes (Ultra-Clear™, Beckman Coulter, Brea, CA), and 10-11mL of lysate was overlaid onto each cushion. Following ultracentrifugation ($100,000 \times g$, 4h, 10°C), supernatants and cushions were aspirated from ultracentrifuge tubes and 200-250 μl calcium and magnesium-free DPBS (CMF-PBS) was added. Samples

were left overnight at 4°C, and pellets were resuspended the next day by pipetting vigorously until observable clumps disappeared. Aliquots of 10µl per sample were diluted in 90µl DPBS for quantitation of virus, and aliquots of concentrated viral suspensions were stored at -80°C.

2.5. RNA extraction and quantitative (q) RT-PCR

Viral (v) RNA was extracted from cell lysates or concentrated virus aliquots using RNeasy Mini kits (Qiagen), using the QiaShredder (Qiagen) first if extracting from ALI culture (Ashraf et al., 2013). vRNA concentrations were determined by qRT-PCR as described previously (Bochkov et al., 2011).

2.6. Osmotic Stability Assay

Clarified lysates from C41 or A16-infected cells were diluted 1:10 in DPBS with added sucrose to produce final concentrations of 0-30% w/v sucrose. Samples (100µL) were incubated for 1 hour at RT with shaking (500 rpm). Aliquots were then diluted 1:10 with DPBS (to decrease the sucrose concentration) and incubated (10min, 37°C) with RNase A (100µg/mL, Qiagen) to remove unprotected vRNA. vRNA content was analyzed by qRT-PCR.

2.7. Thermostability Assay

Clarified lysates from C41 or A16-infected cells were diluted 1:10 in DPBS and then incubated at 4, 37, 46, 55, or 65°C for 1h, followed by 10 min incubation at 37°C in the presence of 100µg/mL RNase A (Qiagen) to remove unprotected vRNA. vRNA content was analyzed by qRT-PCR.

2.8. pH Stability Assay

Clarified cell lysates containing C41, A16, C2, or C15 were diluted 1:10 into various citrate-phosphate buffer combinations (Table 1) thereby exposing virions to a range of pH (7.0, 6.5, 6.0, or 5.5). The samples were incubated at RT with shaking (500 rpm) for 1h, followed by 10min incubation at 37°C with 100µg/mL RNase A (Qiagen), and analysis for vRNA by qRT-PCR.

2.9. Statistics

Statistical significance of collected data was assessed with SigmaPlot programs (version 11.0 Systat Software, Inc., San Jose, CA). Log-transformed values were used for most comparisons. Paired t-tests, one-way ANOVAs, and repeated measures ANOVAs, were used when comparing two groups, three or more groups with one variable, and three or more groups with two variables, respectively. Yield (%) values were determined by dividing the total number of vRNA copies detected per purified sample, by the total number of vRNA copies detected in the starting input lysate.

3. RESULTS

3.1. C41 and C2 yields are lower than C15, following standard concentration protocols

In standard RV concentration protocols (Lee et al., 1995, 1993; Sherry et al., 1986; Wang et al., 1998), lysates from cells transfected with A16, C2, C15, or C41 are incubated with N-lauroylsarcosine to release virus from cell membranes, RNase A to eliminate transfection input RNA, and β -mercaptoethanol to inhibit RNases. Viral particles are then pelleted by centrifugation ($200,000 \times g$, 2h) through a 30% sucrose cushion. Approximately 65% and 60% of A16 and C15 PCR signals were recovered with this procedure, respectively (Figure 1), compared to the RNase-protected signals in starting lysates. However, for C2 and C41, the comparative yields were significantly lower ($<15\%$ and $<5\%$, respectively, $p < 0.001$). Since detergents can adversely affect RV infectivity (Savolainen-Kopra et al., 2012), the first tested protocol variation was to omit N-lauroylsarcosine (Figure 2). The result was a decrease in A16 recovery by approximately 20% ($p < 0.05$), with only a slight increase in C41 yield, from 2 to 5% ($p < 0.01$).

3.2. Effects of stabilizer additions

During purification, other picornaviruses have been stabilized using $MgCl_2$, gelatin, or casein (Behbehani and Lee, 1964; Milo, 1971; Rombaut et al., 1985). Nonfat dry milk (NFDM) or other lipid sources (myristate) have been shown to protect poliovirus and foot and mouth disease virus (FMDV) from thermal inactivation (Dorval et al., 1989; Tomasula et al., 2007). The effects of these compounds on RV-C yield were tested. There was no increase in viral yield following addition of $MgCl_2$, gelatin, casein, or whey to transfected cell lysates, the sucrose cushions, or both (data not shown). In contrast, when 1% NFDM was included in the cushions, the yields of C41 and C2 (the RNase-resistant PCR signals in the resultant pellets) increased to $\sim 60\%$ and $\sim 35\%$ of the input signals, respectively ($p < 0.01$ and < 0.05 , Figure 3). Unfortunately, the final product in both cases consisted of large aggregates of non-viral proteins, lipids, and carbohydrates, and therefore was not suitable for most downstream applications.

3.3. RV-C capsid stability to physical stress

The previous experiments suggested that C2 and C41 capsids were less stable than those of C15 and A16. To test this hypothesis, HeLa cell lysates clarified without detergent containing A16 and C41 capsids were subjected to osmotic, temperature, and pH stresses, and then measured for changes in RNase-protected PCR signals, indicative of particle disruption (Figure 4). Both C41 and A16 responded similarly to osmotic and thermal stress (Figure 4A and B). Both treatments reduced the recovery of protected PCR signals, incrementally, with higher osmotic and temperature shocks. To a degree, the same was true for the pH experiment, except that C41 became sensitive to disruption at pH 6.0, losing 1-log in titer relative to A16 ($p < 0.01$). At a lower pH (5.5), both samples lost PCR signal, indicating common capsid disruption. To determine whether this disparity was common also to C2 and C15 particles, the experiment was repeated with lysates containing these viruses (Figure S1). As with C41, these too had reduced capsid stabilities at pH 6.0. Since C15 yield is high following standard purification, and solutions used in the purification process are

buffered at a pH of ~7.2, RV-C acid lability is an unlikely explanation for the comparatively reduced C2 and C41 yields in standard purification procedures.

3.4. Role of centrifugation in RV-C purification

The above experiments showed there were no overt physical distinctions among C15, C2 and C41 particles that might account for their discrepant recoveries during the standard purification procedure. The NFDM experiment hinted, however, that pelleted aggregates during the centrifugation step might be a source of considerable lost materials. To test this idea, standard purification of C2 was carried out, with or without added NFDM. After centrifugation, the supernatant was decanted and total RNA was extracted from the bottom of the ultracentrifuge tube (Figure 5A). All C2 vRNA signal was detected in this location, even in the absence of NFDM. These pellets resisted resuspension even when they were incubated overnight in CMF-PBS (data not shown). Reasoning these pellets were irretrievably aggregated, the experiment was repeated with lower centrifugal force ($100K \times g$) and commensurately greater time (4h) for sedimentation. Pellet resuspension was again overnight in CMF-PBS (Figure 5B). This simple protocol alteration increased the yield of resuspended C2 and C41 signals by 4-fold and 20-fold respectively compared to the standard protocol ($p < 0.05$ and < 0.01 respectively), while maintaining a similarly high recovery of C15.

3.5. Improved RV-C yields from transfected WisL cells

Preliminary experiments demonstrated greater RV-C replication in transfected WisL cells compared to HeLa cells (data not shown). To capitalize on this observation, the revised centrifugation protocol was applied to virus-containing lysates from these cells (Figure 6). The combined higher initial titers, and improved collection procedures produced excellent C2 and C41 preparations with vRNA concentrations near those observed for parallel C15 preparations (1.7×10^9 , 7.4×10^8 , and 5.2×10^9 vRNA copies/ μ l, respectively).

3.6. Infectivity of purified C41 and C2 to differentiated HBECs

C41 and C2 suspensions prepared with the improved protocol were tested for binding and infectivity to ALI cultures of HBECs (Figure 7). HBEC ALI cultures were inoculated with 1×10^6 units of purified virions per well or with medium alone. The amplification of C2 was greater than that of C41, but both viruses increased their vRNA signals by nearly 2 logs, over 24h, compared to the amount of cell associated signal at 3 hours post-inoculation (hpi).

4. DISCUSSION

The RV-C are clinically important viruses that were first discovered in 2006. Research on these viruses has been hampered by difficulties in producing adequate preparations of purified viral particles. The standard RV purification protocol was adapted from methods used for cardioviruses (Rueckert and Pallansch, 1981). While successful for multiple RV-A and B types, and for recombinant virus derived from the C15 cDNA, this protocol was unable to recover high yields for other RV-C types (Bochkov et al., 2011; Lee et al., 1995, 1993; Nakagome et al., 2014; Sherry and Rueckert, 1985). Only minor differences were found in capsid stability between those RV refractory to purification (e.g., C2 and C41), and

those that were purified successfully (e.g., C15 and A16). However, it was observed that the former formed tight aggregates following high-speed centrifugation, and these pellets were resistant to resuspension. By reducing the centrifugal force and increasing the spin-time, the resulting pellets could be resuspended, which led to significantly improved virus recovery. Using the revised protocol, C41 and C2 yields were comparable to those attained with C15 or the standard RV-A and RV-B types.

Additional variables were tested as part of this protocol. The removal of detergent negatively affected the recovery of A16, but did not substantially impact C41. This may reflect differential association of newly formed particles with HeLa cell membranes. Newly formed A16 may become trapped in complexes with ICAM-1, highly expressed on HeLa cell membranes, which are liberated by incubation with detergent. Since HeLa membranes lack receptors for C types and do not bind these viruses, adding detergent did not improve the yield of C types. In addition, none of the putative stabilizing agents tested here had a positive impact on virus recovery. NFDm, while improving purification yield, produced large aggregates inadequate for downstream applications. Although some of these agents are useful for other picornavirus purifications, they do not add much to efficiency of RV yields (Behbehani and Lee, 1964; Milo, 1971; Rueckert and Pallansch, 1981; Tomasula et al., 2007).

Instead, the most important observation described here was that ultracentrifugation of C2 and C41 could lead to tight pellets that were resistant to resuspension. This suggests that the physical properties of some RV-C may promote aggregation. Gassilloud and Gantzer made similar observations with poliovirus, and further noted that incubating poliovirus in a buffer with pH near its isoelectric point (pI) promoted the highest degree of aggregative and adherent properties (Gassilloud and Gantzer, 2005).

Relative to the original protocol, a simple reduction in centrifugal force, and increase in spin time had a large effect on viral yield (~0.5-1.5 log). Furthermore, the use of WisL cells as targets for RNA transfection increased the apparent amount of virus produced per cell by ~1 log. These larger starting yields, when coupled to the reduced-g procedures, converted directly into highly concentrated and infectious samples for all tested RV-C.

In conclusion, the modified RV purification protocol enables production of multiple RV genotypes, while maintaining excellent infectivity. The three RV-C genotypes examined have slightly different physical properties, and additional experiments may reveal whether these differences influence virus binding, replication, or transmission. The protocol revisions and stability data presented in this work will assist and expedite RV-C research related to antiviral development, characterization of RV-C physical and immunological properties, and exploration of the RV-C lifecycle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Standard purification methods have very low infectious Rhinovirus C (RV-C) yields.
- The RV-C have comparable osmotic and thermal stability to the RV-A.
- The RV-C capsid may be more sensitive to pH than the RV-A.
- Drop in centrifugal force and increased spin-time recovered high yields of RV-C.
- RV-C purified with revised method were infectious on respiratory epithelium.

Figure 1. Variable RV-C yield with standard purification protocol

HeLa cell lysates transfected with either C2, C41, C15, or A16 were purified using the standard protocol (n=3) (16,17,32,35). *** p -value <0.001.

Figure 2. Effects of detergent on virus yield

Cell lysates were treated (+) or not (-) with N-lauroylsarcosine. RNase protected virus signals recovered by each protocol was recorded (n=3). *, ** p -value <0.05 and <0.01, respectively.

Figure 3. Effect of NFDM on virus yield

Following transfection of HeLa cells with recombinant transcripts, C41- or C2-containing clarified cell lysates were fractionated by pelleting through a 30% sucrose cushion containing PBS with 1% nonfat dry milk (NFDM), or PBS alone (n=4 and n=3 for C41 and C2 experiments, respectively). *, ** p -value < 0.05 and <0.01, respectively.

Figure 4. Response of RV-C41 to physical stress

HeLa cell lysates containing newly synthesized A16 or C41 were subjected to conditions intended to induce potential capsid disassembly. The assays measured gain/loss of vRNA PCR signal, following treatment with RNase A. To assay osmotic stress factors, (A), lysates were incubated for 1h, with shaking at RT in PBS containing 0, 1, 3, 10, or 30% sucrose (w/v, n=3). To assay thermal stress (B), lysates were incubated for 1h at 4, 37, 46, 55, or 65°C (n=5). To assay pH stress (C), lysates were diluted into citrate-phosphate buffers with the indicated pH, and then incubated for 1h with shaking (n=3). *, **, *** p -value <0.05, <0.01, and <0.001, respectively.

Figure 5. RV yield with modified centrifugation protocol

(A) Following purification of C2 through 30% sucrose with (NFDM) or without (PBS) NFDM, total RNA was extracted and quantified via qPCR to determine the total RNase-protected material in the pellet prior to resuspension (n=3). (B) HeLa cell lysates containing C41, C2, or C15 were loaded over 30% sucrose cushions and subjected to centrifugation at either 200K × g for 2h, or 100K × g for 4h (n=3). The resultant pellets were quantified for RNase-resistant PCR signals. N.S., *, *** *p*-value >0.05, <0.05, and <0.001, respectively.

Figure 6. Purification of C2, C41, and C15 from WisL cells, using the revised protocol

Following transfection of WisL cells with C2, C41, or C15 RNA, lysates were harvested (~10mL each) and pelleted (100K × g for 4h) through 30% sucrose cushions and then resuspended overnight in CMF-PBS (250μl) (black bars, n=3). Gray bars indicate yields obtained with standard, previous purification procedures, from HeLa cells, resuspended in the same volume (n=3). *, *** *p*-value <0.05 and <0.001, respectively.

Figure 7. Infectivity of purified RV-C preps

Cultures of differentiated epithelial cells (ALI) were inoculated with 10^6 vRNA copies/well of C41 or C2 produced using the revised protocol. BEGM media alone served as a control (mock). vRNA was assayed by qPCR at 3 hours post-inoculation (3hpi) and again at 24hpi to determine cell-associated virus prior to and post viral replication, respectively (n=1). vRNA was not detected in mock-infected samples. Abbreviations: B.D., below detection.

Table 1
Citrate-Phosphate Buffer Stock Combinations

pH	X mL 0.1M Citric Acid	Y mL 0.2M Na ₂ HPO ₄
5.5	43.1	56.9
6.0	36.9	63.2
6.5	29.0	70.9
7.0	17.7	82.4