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Sequencing human rhinoviruses: Direct sequencing versus plasmid cloning

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A B S T R A C T

Human rhinoviruses (RV) are associated with the majority of viral respiratory illnesses in infants, children and adults. Over the last several years, researchers have begun to sequence the many different species and strains of RV in order to determine if certain species were associated with increased disease severity. There are a variety of techniques employed to prepare samples for sequencing. One method utilizes plasmid-cloning, which is expensive and takes several hours to complete. Recently, some investigators have instead used direct sequencing to sequence RV strains, allowing for omission of the time- and labor-intensive cloning step. This study formally compares and contrasts the sequencing results obtained from plasmid-cloning and direct Sanger sequencing of a 500 base pair PCR product covering the VP4/VP2 region of RV. A slightly longer sequence (by 65 base pairs on average) was obtained when specimens were plasmid-cloned, and the sequences were 86% similar. After trimming the extra base pairs from the cloned sequences, the sequences were 99.7% identical. Overall success of directly sequencing samples was similar to that of cloning, 5% on average failed for each technique. Therefore, in many instances, directly sequencing samples may be considered in lieu of the more expensive and time-consuming plasmid-cloning technique.

1. Introduction

Human rhinoviruses (RV) are medically important pathogens that are associated with upper and lower respiratory infections (Jartti et al., 2004; Lemanske et al., 2005; Miller et al., 2009; Bizzintino et al., 2011; Cox et al., 2013; Linder et al., 2013). Advancement in molecular sequencing methods over the last several years has allowed researchers to genotype hundreds of strains of RV. This led to the discovery of a new species of RV in 2006, now known as RV-C (Lamson et al., 2006; Lau et al., 2007; Lee et al., 2007a; McErlean et al., 2007). Certain species of RV may be associated with varying disease severity. For example, RV-C is associated frequently with lower respiratory infections and asthma exacerbations (Jartti et al., 2004; Lemanske et al., 2005; Miller et al., 2009; Bizzintino et al., 2011; Cox et al., 2013; Linder et al., 2013). In addition, seasonal variation of the RV species differs (Annamalay et al., 2012; Lee et al., 2012; Linder et al., 2013; Pierangeli et al., 2013). Therefore, it is important for researchers to have the ability to identify the strains and species of RV. Currently there are no vaccines available for the >150 types of RV. However, if a treatment or vaccine was available for the more pathogenic strains, rapid identification of the strain of RV infecting a patient could be necessary.

To successfully sequence RV, the genomic RNA (or cDNA) must be amplified to produce an adequate copy number. Initially, researchers cloned RV gene fragments amplified by reverse transcriptase (RT)-PCR into plasmid vectors in order to amplify the sequence and allow for successful cloning (Lee et al., 2007b; Pierangeli et al., 2007; Huang et al., 2009; Olenec et al., 2010; Xiang et al., 2010; Miller et al., 2011; Linder et al., 2013). Plasmid-cloning was desirable because it allowed for amplification of very small quantities of cDNA from samples. This technique was also useful in amplifying large portions of unknown strains of RV genome. However, recently researchers shifted to directly sequencing the cDNA product after purification. Most articles published within the last

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year that sequenced RV utilized this method (Daleno et al., 2013; Garcia et al., 2013; Miyaji et al., 2013; Pilorge et al., 2013). Others utilized direct sequencing in earlier studies as well (Blomqvist et al., 2009; Kneider et al., 2009; Wisdom et al., 2009; Mizuta et al., 2010; Watanabe et al., 2010). Plasmid-cloning typically takes three days to complete due to incubation times; however, direct sequencing is conducted immediately after RT-PCR amplification and purification. Due to labor costs alone, researchers may have preferred direct sequencing to save both time and money. However, direct sequencing of RT-PCR product has not been rigorously compared to plasmid-cloning to the authors knowledge. It is not known if the quality of the resulting sequences is similar in terms of which nucleotides are observed, or if the two techniques have equivalent success rates. In this study, RV fragments that were amplified and plasmid-cloned were compared to RV fragments that were amplified and directly sequenced from the same sample. This allowed for comparison of cost, technique, and quality of results between the two methods to determine the most efficient way to sequence many RV samples.

Table 1
Comparison of time and material costs between plasmid-cloned and directly sequenced samples. USD = United States Dollars.

	Plasmid-cloning	Direct sequencing
Number of steps	9 Run gel Excise and extract bands Ligate Transform Grow on agar plates Amplify colony Mini prep Digest Run gel	3 Run gel Nanodrop ExoSap-IT[a]
Active time	10 h	2 h
Total time	3 days	3 h
Cost (materials)	14 USD/sample	2 USD/sample
Cost (labor $20/h)	200 USD	40 USD
Total cost (16 samples)	424 USD	72 USD

[a] VANTAGE Vanderbilt Sequencing Core purified samples with ExoSap-IT at a cost of $0.70 per sample.

2. Methods

2.1. Sample collection and processing

Samples utilized for testing were nasal aspirates collected from a cohort of very low birth weight (<1500 g) premature infants (<32 weeks gestational age) in Buenos Aires, Argentina, which had been approved by the Internal Review boards at Vanderbilt University, USA, Pediatric Hospital de J.P. Garrahan, and the Hospital Materno Infantil Ramón Sarda. Parental consent was obtained for enrollment and sampling, and families could refuse sampling or withdraw from the study at any time. Patients were enrolled from June 2011 to October 2012 during the infants' first year of life. Samples were collected using sterile saline with no additives and were immediately frozen until shipment on dry ice to the United States. Total nucleic acids were extracted using the Roche Magna Pure system (kit # 3038505001). Conventional reverse transcriptase (RT)-PCR was then conducted two separate times (once for the plasmid-cloning and once for the direct sequencing) using Qiagen One step RT kit (#210212), including positive and negative controls with each run. Primers (Savolainen et al., 2002) encompassed the VP4/VP2 region and were as follows: RV-Forward GGGACCRACTACTTTGGGTGTCCGTGT;RV-Reverse GCATCWGGYARYTTCCACCACCADCC, where there were four wobble primers W (A+T) Y (C+T), R (A+G), and D (A+G+T). After processing, samples were sent to the Vanderbilt sequencing core and Sanger sequencing was conducted. Samples were sequenced in one direction. For cloned samples, a T7 primer was used. For directly sequenced samples, the RV-forward or RV-reverse primer was provided with the samples for sequencing.

2.2. Plasmid-cloning

After RT-PCR was completed, 10 μl of each sample was run on a 1% agarose gel. Positive bands (approximately 525 base pairs long) were excised with a clean scalpel and DNA was extracted using the Qiagen Qiaquick gel extraction kit (#28706). Supplemental Multimedia Component 1 illustrates an example of a RT-PCR gel. Running the gel confirmed that there was one band of the correct size. By excising the band, no sample was discarded and only the target nucleotides were processed. Samples were then ligated into the pGEM-T Easy Vector (Promega #A1360), and transformed into DH5 alpha (ampicillin resistant) competent *E. coli* cells. Cells were shaken at 37 °C for 90 min and then plated onto ampicillin-infused agar plates coated with 100 mM IPTG (isopropyl-beta-D-thiogalactopyranoside) and 50 mg/ml of Xgal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside). On day two, colonies that were successfully ligated and transformed (white colonies) were picked with a sterile toothpick and placed into 2 ml of broth containing 4 ul of 100 mg/ml ampicillin. Cultures were shaken at 37 °C overnight. On day three, the plasmids were extracted from the bacteria using the Qiagen Miniprep kit (#74106). Five microliters of each sample was then digested using the EcoR1 restriction digest enzyme and run on a 1% agarose gel to confirm that the correct size of DNA fragment was incorporated into in the plasmid (approximately 525 base pairs). If the RV sequence was not observed in the plasmid after restriction digest, another colony was picked and reprocessed. Supplemental Multimedia Component 2 details the cloning protocol used in this laboratory. The whole procedure requires approximately 10 h of active labor to process 16 samples, and three days of processing due to incubation times. In this laboratory the material cost (including reagents) to prepare one sample for sequencing was approximately 14 US dollars (USD), not including the cost of the initial RT-PCR, the sequencing costs at the VANTAGE Vanderbilt Sequencing Core, or labor. If labor costs were included, (at a cost of 20 USD an hour for labor), the total was 424 USD to process 16 samples.

2.3. Direct sequencing

After RT-PCR was conducted, 10 μl of each sample was run on a 1% agarose gel to confirm a positive band. Positive samples were then quantified using a Nanodrop (Thermo Scientific) and diluted with sterile water to reach a final concentration of approximately 50 ng/μl. Samples were sent to the VANTAGE Vanderbilt Sequencing Core to be treated with ExoSap-IT (Affymetrix #78200) for removal of PCR contaminants, and then sequenced. The cost of ExoSap-IT at the VANTAGE Vanderbilt Sequencing Core was $0.70 per sample. Thus, the material cost (including reagents) of preparing one sample for sequencing was approximately 2 USD, again not taking into account the cost of RT-PCR, the sequencing costs at the VANTAGE Vanderbilt Sequencing Core, or labor. The time required to set up the experiment, run the gel, and prepare the samples for sequencing was approximately 2 h, with labor costs included (20 USD/h) the total cost was 72 USD to process 16 samples. Table 1 shows a comparison of the time and material costs between the two sequencing methods. VP4/VP2 sequences were submitted to GenBank (accession numbers KJ620336–KJ620367).