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Human airway epithelial cell culture to identify new respiratory viruses: Coronavirus NL63 as a model

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Propagation of new human respiratory virus pathogens in established cell lines is hampered by a lack of predictability regarding cell line permissivity and by availability of suitable antibody reagents to detect infection in cell lines that do not exhibit significant cytopathic effect. Recently, molecular methods have been used to amplify and identify novel nucleic acid sequences directly from clinical samples, but these methods may be hampered by the quantity of virus present in respiratory secretions at different time points following the onset of infection. Human airway epithelial (HAE) cultures, which effectively mimic the human bronchial environment, allow for cultivation of a wide variety of human respiratory viral pathogens. The goal of the experiments described here was to determine if propagation and identification of a human respiratory virus may be achieved through inoculation of HAE cultures followed by whole transcriptome amplification (WTA) and sequence analysis. To establish proof-of-principle human coronavirus NL63 (HCoV-NL63) was evaluated, and the first visualization of HCoV-NL63 virus by transmission electron microscopy (TEM) is reported. Initial propagation of human respiratory secretions onto HAE cultures followed by TEM and WTA of culture supernatant may be a useful approach for visualization and detection of new human respiratory pathogens that have eluded identification by traditional approaches.

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1. Introduction

The detection of human respiratory virus pathogens can be challenging due to difficulty in obtaining high titer clinical specimens, and the inefficiency of propagating the virus in established cell lines. Viruses that replicate readily in culture and induce dramatic cytopathic effect (CPE), such as influenza viruses, respiratory syncytial viruses, adenoviruses, rhinoviruses and coronaviruses responsible for common colds (229E and OC43) were identified and characterized by electron microscopy in the 1960s. However, these efficiently propagating viruses likely represent only a fraction of

the viruses that cause significant clinical disease (Iwane et al., 2004; Jartti et al., 2004; Juven et al., 2000). In recent years, viral respiratory pathogens such as human metapneumovirus (van den Hoogen et al., 2001) and human coronavirus NL63 (HCoV-NL63) (van der Hoek et al., 2004) that propagate slowly in standard cell lines have been identified using molecular approaches to amplify novel sequences from infected cell lines. However, the optimal propagation of a human respiratory virus and detection of virus-infected cells may still be a roadblock for identification of novel pathogens. A culture system which faithfully mimics human airways may alleviate some of these challenges. Therefore, human airway epithelial (HAE) cells were evaluated as a culture system for the initial propagation of a human respiratory virus, followed by visualization of the virus by transmission electron microscopy (TEM) and use of a random amplification approach to detect viral sequences.

HAE cultures are derived from primary bronchial epithelial cells isolated from the airways of human lung donors or patients undergoing lung transplantation, and have been used extensively to study the biology of respiratory epithelium (Fulcher et al., 2005). Primary bronchial epithelial cells are harvested from the inner lining of airways and are cultured on porous supports, initially submerged in

Abbreviations: HCoV-NL63, human coronavirus NL63; WTA, whole transcriptome amplification; SARS-CoV, severe acute respiratory syndrome coronavirus; CPE, cytopathic effect; TEM, transmission electron microscopy; HAE, human airway epithelium.

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medium. After the cells grow to form a confluent monolayer, the medium is removed from the apical side, creating an air–liquid interface culture. The primary cells then replicate and differentiate to recapitulate the pseudostatified epithelial morphology found in the human airway. Mature differentiated HAE cultures can be maintained for up to 2 months and contain mucus-producing goblet cells and non-ciliated and ciliated epithelial cells, which have

been shown to be ideal for propagation of a wide range of human respiratory pathogens, including influenza virus (Thompson et al., 2006), parainfluenza viruses (Zhang et al., 2005), respiratory syncytial virus (Zhang et al., 2002), adenovirus (Zhang et al., 2002), and severe acute respiratory syndrome coronavirus (SARS-CoV) (Sims et al., 2006). Propagation of human viral pathogens in clinical specimens on HAE cultures may provide an optimal environment.

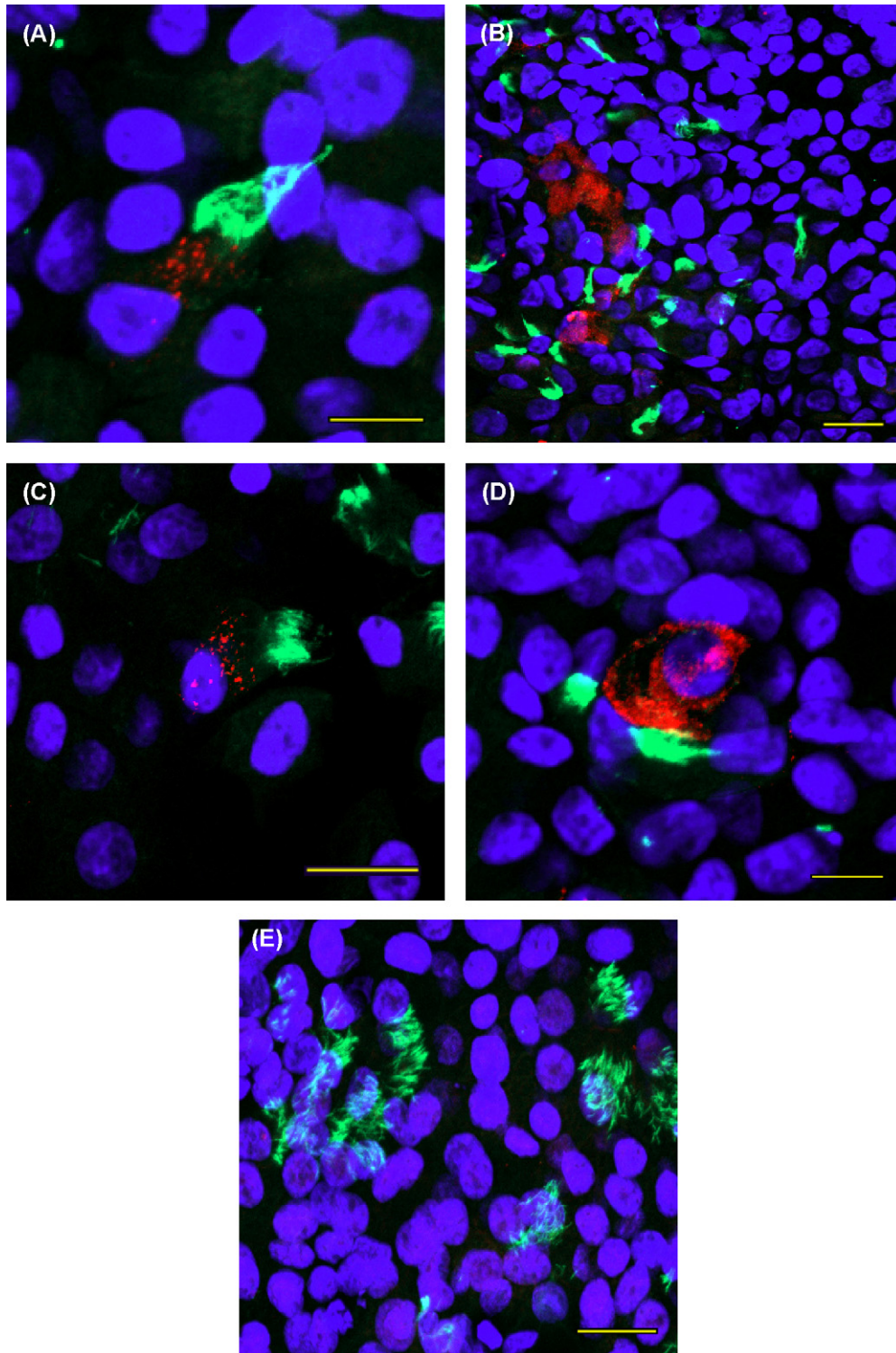


Fig. 1. Detection of HCoV-NL63 in the ciliated epithelium of HAE cultures by immunofluorescence. HCoV-NL63-infected (A–D) or mock-infected (E) HAE cultures were stained with anti-HCoV-NL63 replicase (red), anti-tubulin (green) and DAPI (blue) at 72 h post-infection and imaged by confocal microscopy. (A and D) Bar, 10 μ m. (B, C and E) Bar, 20 μ m.

Therefore, HAE cultures may be a useful culturing technique for virus discovery research. To establish proof-of-principle for this concept, the replication of HCoV-NL63 in HAE cultures was studied.

HCoV-NL63 was identified as a virus that propagated inefficiently in standard cell culture (van der Hoek et al., 2004). Researchers noted that a clinical specimen obtained from a pediatric patient hospitalized with a respiratory tract infection induced a low level of CPE after 8 days of incubation in a monkey kidney cell line, LLCMK2 cells. Using a novel molecular amplification, van der Hoek et al. (2004) were able to amplify, sequence and identify a novel coronavirus, designated HCoV-NL63, as the infectious agent. Interestingly, this virus was also identified in respiratory secretions from children with respiratory symptoms by two additional independent research groups (Esper et al., 2005; Fouchier et al., 2004). In addition to causing upper respiratory infection, HCoV-NL63 is responsible for croup in children (van der Hoek et al., 2005), and is distributed worldwide (Arden et al., 2005; Bastien et al., 2005; Suzuki et al., 2005; Vabret et al., 2005). Identification of HCoV-NL63 has added to knowledge regarding human respiratory viruses, and will allow for development of a diagnostic test and study of potential therapies for this infection.

Surveillance studies of children with respiratory illnesses indicate that in 22–39% of cases no specific etiologic agent is detected (Iwane et al., 2004; Jartti et al., 2004). It is likely that many human respiratory viruses remain unidentified because they do not propagate efficiently using standard tissue culture cell lines (Allander et al., 2005). Using HCoV-NL63 a novel variation of virus propagation in HAE cultures followed by visualization of the virus by TEM and identification of the viral genome in culture supernatants using a sequence-independent amplification technique is described.

2. Materials and methods

2.1. Virus and cells

HCoV-NL63 and LLCMK2 cells were obtained from Dr. Lia van der Hoek (University of Amsterdam, The Netherlands). The virus was propagated in LLCMK2 cells as previously described (Chen et al., 2007) and cytopathic effect was evident after 5–6 days in culture. Supernatant collected from inoculated cells contained approximately 2–10 TCID₅₀ per ml.

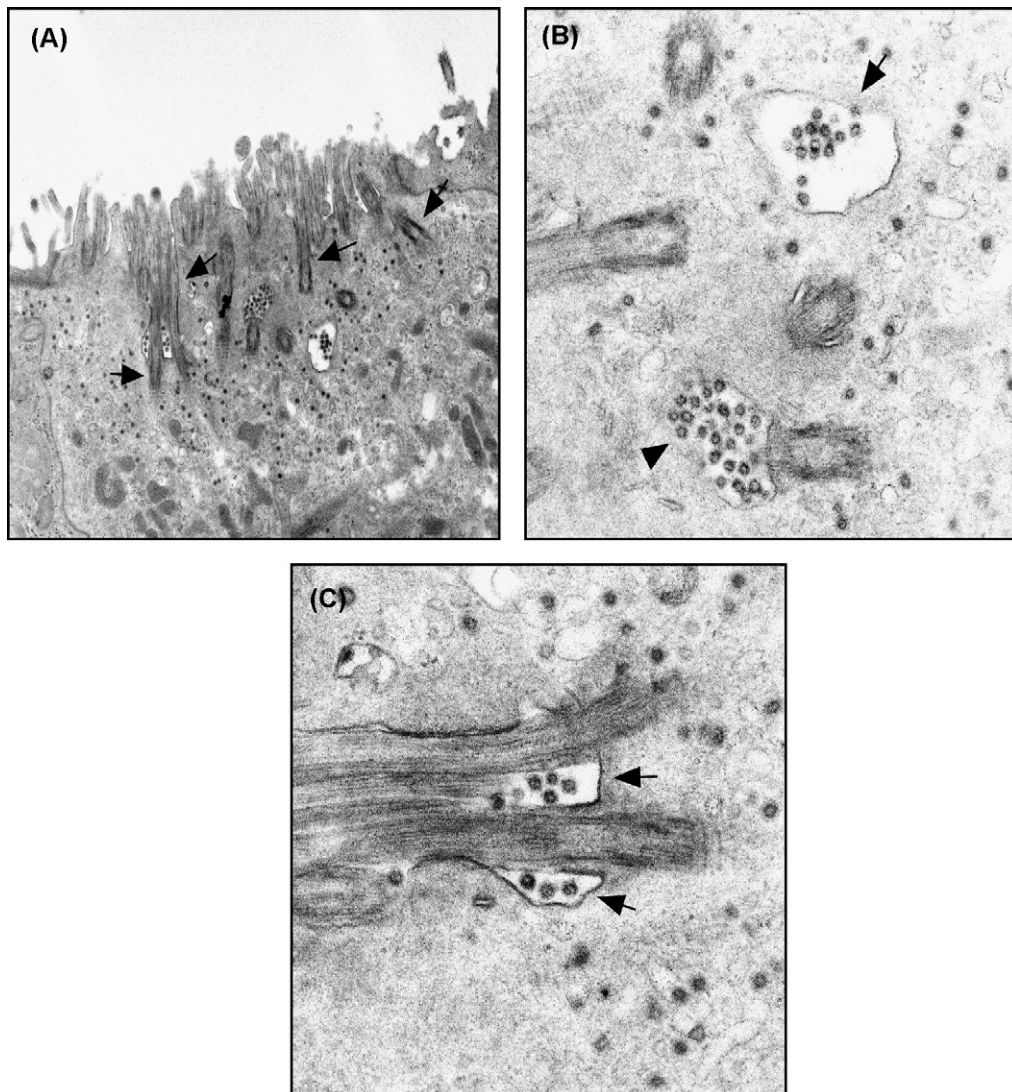


Fig. 2. Detection of HCoV-NL63 using TEM. (A) HCoV-NL63 replication and budding into vesicles occurs in ciliated cells. The cilia (arrows) are tangentially sectioned; $\times 39,000$. (B) HCoV-NL63 virions in a clear Golgi vacuole (arrow). A second vacuole (arrowhead) is at the base of a cilium. Single virions in small vacuoles are detected in the background; $\times 126,000$ (C) Extracellular virions surround the base of two cilia (arrows). Single intravacuolar virions are in the background; $\times 146,000$.

Human airway epithelial cultures were generated from primary human bronchial epithelial cells (IRB approval number LU#200155) following established procedures (Fulcher et al., 2005). Cultures were inoculated with 0.2 TCID₅₀ of HCoV-NL63 and incubated at 33 °C. Whole mount cultures were fixed for immunofluorescence or TEM studies at the times indicated.

2.2. Immunofluorescence analysis of human airway epithelial cell cultures

HCoV-NL63 anti-nsp3 anti-serum (rabbit) was generated as previously described (Chen et al., 2007). HAE cultures were infected with 100 μl of HCoV-NL63 virus stock. At 24, 48, and 72 h post-infection cultures were fixed, permeabilized, and stained with the indicated antibody. HAE culture supernatant was recovered in 200 μl of F12 media. Cells were washed three times with PBS, fixed with 4% paraformaldehyde for 10 min at 4 °C, permeabilized with 0.2% Triton X-100 for 8 min at room temperature, and subsequently washed in PBS. To block nonspecific binding, the fixed cells were incubated with histoblock [5% normal goat serum, 1% bovine serum albumin, 1% teleost gelatin in PBST (PBS with 0.05% Tween 20)] for a minimum of 1 h at 4 °C. HCoV-NL63 anti-nsp3 antisera was diluted 1:1000, and mouse monoclonal anti-β tubulin IV (Sigma) was diluted 1:400 in diluent (1:3 dilution of histoblock in PBST), and incubated with fixed cells overnight at 4 °C. Cells were washed three times with diluent at 4 °C with agitation. Anti-rabbit immunoglobulin (Ig heavy and light chain) Alexa Fluor-568 secondary antibody (Invitrogen), and anti-mouse immunoglobulin (Ig heavy and light chain) Alexa Fluor-488 secondary antibody (Invitrogen) were diluted 1:400 in diluent, and incubated with cells overnight at 4 °C. To detect nuclear DNA cells were stained with DAPI (stock solution at 5 mg/ml) at a 1:1000 dilution in PBS for 10 min at room temperature. Cultures were then washed three times with PBST and mounted with PermaFluor mounting medium (Thermo Scientific). Immunofluorescence staining was visualized using a Zeiss LSM-510 confocal microscope at the Loyola University Medical Center Core Imaging Facility.

2.3. Transmission electron microscopy

HAE cultures were fixed at 72 h post-infection in 4.0% glutaraldehyde (0.1 M sodium cacodylate buffer, pH 7.4) overnight at 4 °C and then postfixed with 1.0% osmium tetroxide for 1 h. Following serial ethanol and propylene oxide dehydration, samples were embedded in EMBED-812 (Electron Microscopy Sciences, Hatfield, PA). Sectioning and poststaining were performed using standard methods (Bozzola and Russell, 1998). Samples were examined using a Zeiss EM10 transmission electron microscope operating at 60 kV.

2.4. Whole transcriptome amplification

Apical washes from mock and HCoV-NL63-infected HAE cultures were collected and centrifuged for 10 min at 13,500 rpm to remove cell debris. The cell-free supernatant was treated with 5 U of *DNase I* enzyme (Ambion) for 45 min at 37 °C. The enzyme was heat inactivated by adding EDTA to a final concentration of 5 mM and incubating the solution for 10 min at 75 °C. Total RNA was isolated using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. RNA was reverse transcribed and amplified using TransPlex® Whole Transcriptome Amplification Kit (Sigma) according to the manufacturer's instructions. DNA was purified using Wizard® PCR Preps DNA Purification System (Promega), cloned into TOPO TA vector (Invitrogen), and transformed into TOP10 *E. coli* cells (Invitrogen). Plasmid DNA was isolated, digested with *EcoRI*

(Fermentas), and digested DNA was analyzed on a 1.5% agarose gel to visualize the size of the inserts. Plasmid DNA positive for insert was sequenced by the University of Chicago Cancer Research Center DNA Sequencing Facility using version 3.0 BigDye GTP sequencing reaction kit (Applied Biosystems).

3. Results

3.1. Immunofluorescence detection of HCoV-NL63 in human airway epithelial cultures

Previous studies have shown that HAE cultures can be used to efficiently propagate human respiratory viruses (Sims et al., 2006; Thompson et al., 2006; Zhang et al., 2002, 2005). However, it is unclear whether viruses that replicate less efficiently in standard cell culture, such as HCoV-NL63 (Schildgen et al., 2006), would propagate efficiently in HAE cultures. Therefore, HAE cultures were inoculated with 0.2 TCID₅₀ of HCoV-NL63 and incubated at 33 °C for 72 h. Cultures were then fixed and stained with anti-serum directed against replicase product expressed in HCoV-NL63-infected cells

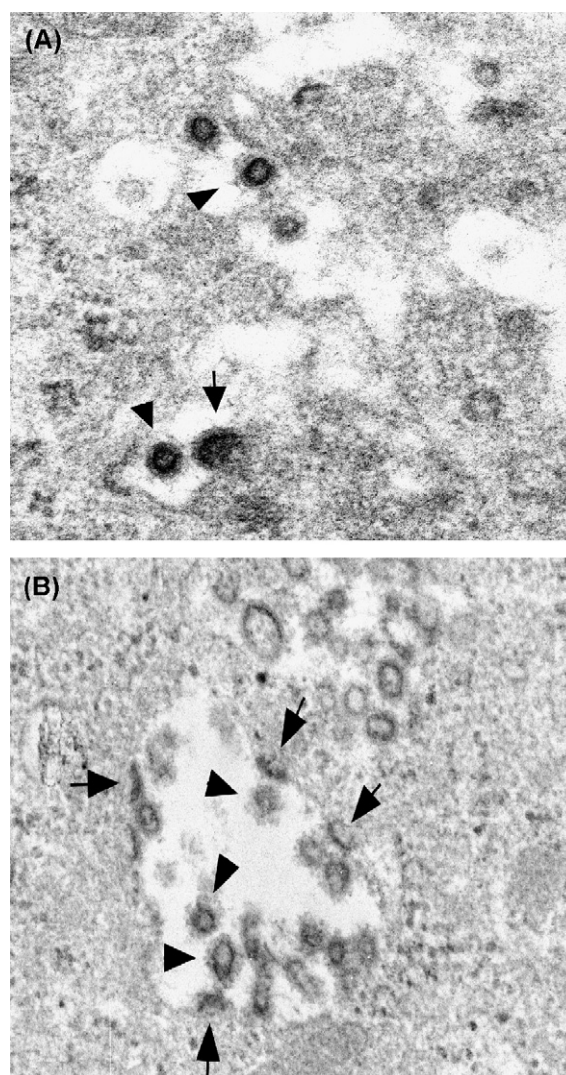


Fig. 3. Budding of HCoV-NL63 into smooth-walled vesicles. (A) A budding virion is seen in a vacuole (arrow). Two other virions (arrowheads) have visible spikes, characteristic of members of the *Coronaviridae*; $\times 245,000$. (B) A vacuole with several budding virions (arrows) and free particles covered with spikes (arrowheads); $\times 175,000$.

(Chen et al., 2007). HCoV-NL63 replication was detected in the ciliated bronchial epithelial cells in the HAE cultures (Fig. 1). Cilia were stained with anti-tubulin antibody (green), nuclei were stained with DAPI (blue), and HCoV-NL63 was stained with antibodies directed against replicase product nonstructural protein 3 (nsp3) (red). HCoV-NL63 replicase protein was detected exclusively in the cytoplasm, with punctate, perinuclear staining evident in discrete foci in the culture. Over a time course of 5 days, spread of infectious virus was detected by visualizing the broader distribution of the antigen positive cells and by passaging cell-free supernatant to fresh cultures and detecting HCoV-NL63 virus at low titer (10–100 TCID₅₀/ml) 48–102 h post-infection (data not shown). This is in stark contrast to SARS-CoV, which replicates to high titer (10⁸ pfu/ml) in both VeroE6 cells and HAE cultures (Sims et al., 2005). These results indicate that HCoV-NL63 productively infects ciliated cells. In addition, there was minimal apparent cytopathicity observed in the HCoV-NL63-infected HAE cultures. Similar immunofluorescence results were obtained in three independent experiments. Therefore, this HCoV-NL63 model system manifest-

ing low levels of viral replication and virus particle production was ideal to test whether TEM coupled with random amplification of infected cell supernatant could identify a virus that was produced at only low titer in this system.

3.2. Transmission electron microscopy of HCoV-NL63 in human airway epithelial cultures

To determine if virus particles could be visualized in HCoV-NL63-infected HAE cells, mock-infected and HCoV-NL63-infected HAE cultures were analyzed using TEM. All HAE cultures contained a typical mixture of ciliated and non-ciliated bronchial cells, and mucus-producing goblet cells. The mock-infected HAE cultures did not contain HCoV-NL63 virus particles, and cell degeneration and necrosis was extremely rare (data not shown). In HCoV-NL63-infected cultures, virus particles were readily detected by careful examination of the ciliated cells (Fig. 2A–C). Viral morphogenesis occurred in the apical, supranuclear, Golgi/RER region of the cell cytoplasm. Virus particles, with club-shaped spikes, budded

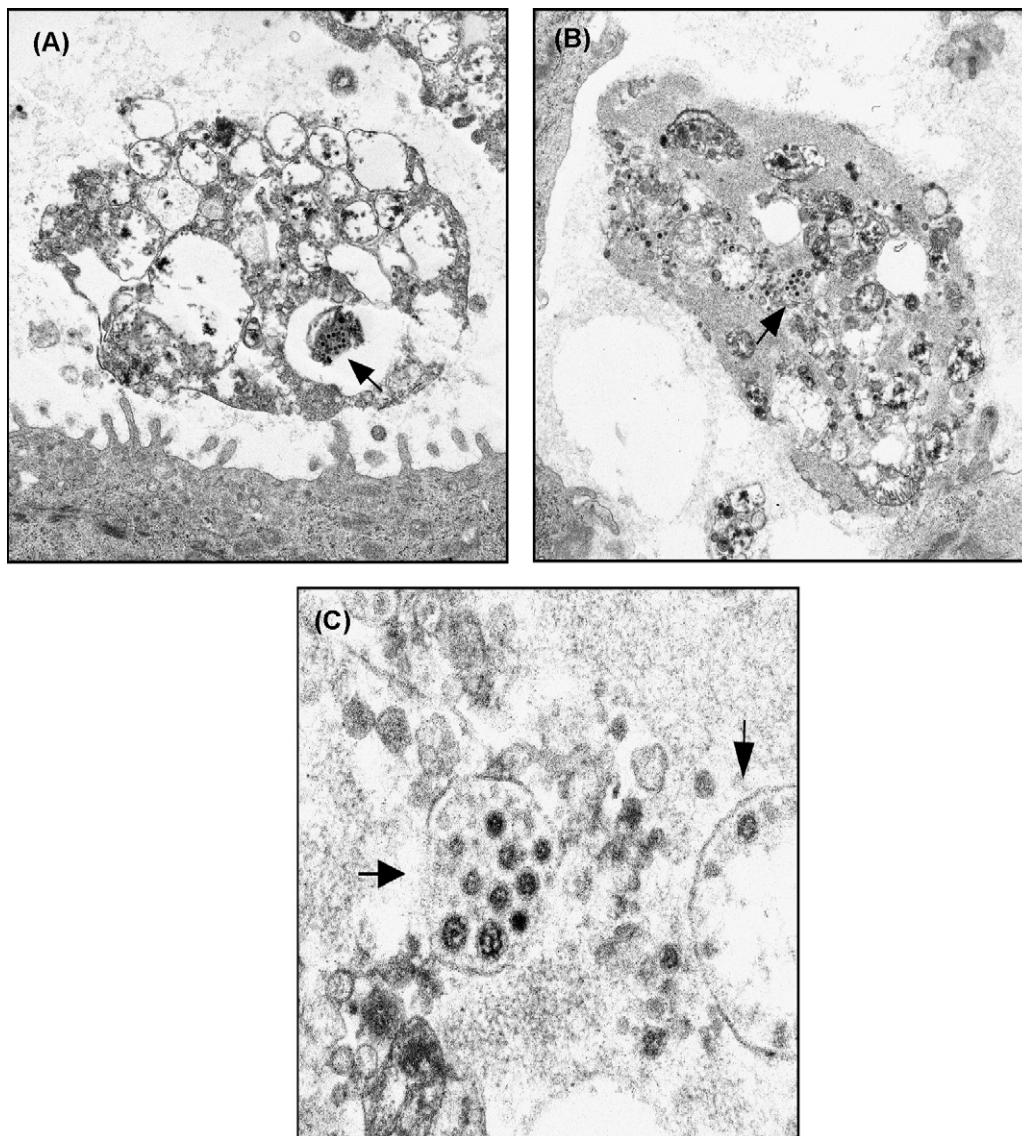


Fig. 4. Evidence of lytic infection by HCoV-NL63. (A) Section of a necrotic cells on the surface of an HAE culture. One of the vacuoles contains virions (arrow); $\times 33,000$. (B) An electron-dense apoptotic cell with vesicles containing virions (arrow) is present within the HAE cell layer; $\times 35,000$. (C) Enlargement of (B), showing the cell contains vacuoles (e.g. arrows) with virions; $\times 184,000$.

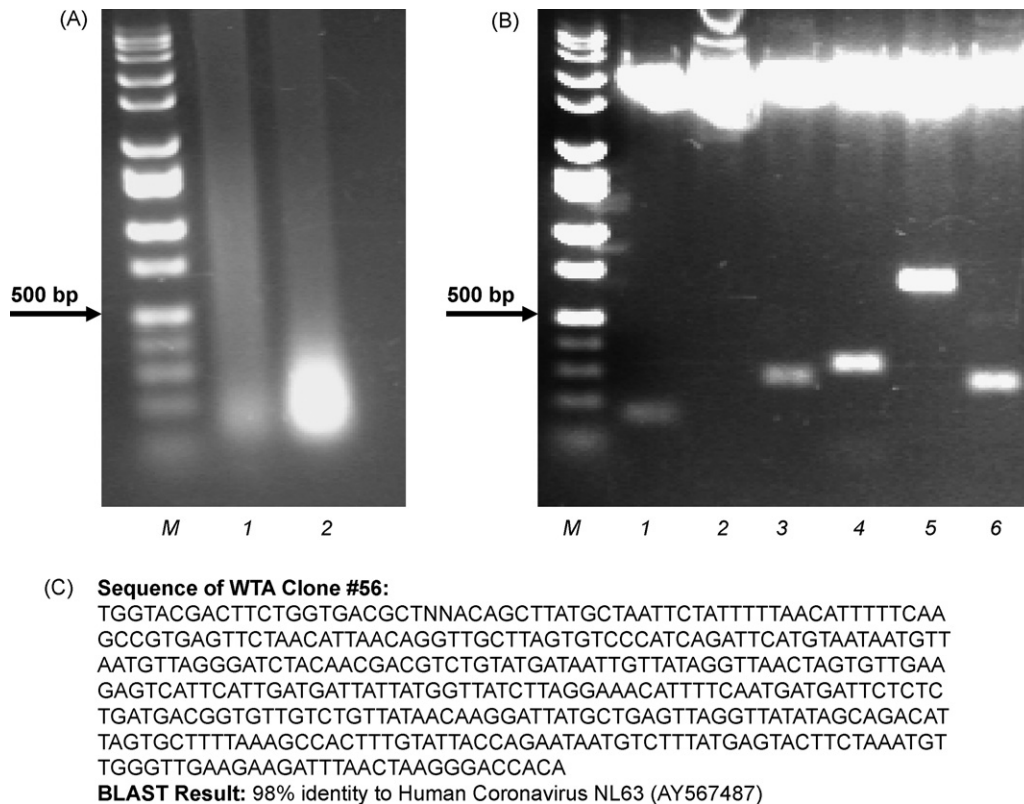


Fig. 5. Analysis of products of WTA reaction products generated from RNA isolated from the supernatant of mock-infected and HCoV-NL63 inoculated HAE cultures. (A) Ethidium bromide stain of WTA products from mock-infected (lane 1) and HCoV-NL63-infected (lane 2) cells; (B) analysis of products generated by restriction enzyme digestion (EcoRI) of cloned WTA products separated by electrophoresis on a 1.5% agarose gel and visualized by staining with ethidium bromide. (C) Sequence of clone #56 identified by BLAST as 98% homologous to HCoV-NL63.

into smooth-walled vesicles (Fig. 3A and B). The typical HCoV-NL63 spherical virion measured from 75 to 115 nm in size, with surface spikes projecting 10–20 nm from the viral envelope. This observed morphology is consistent with the average 100 nm diameter and 20 nm spikes of other viruses within the *Coronaviridae* (Lai et al., 2007). The HCoV-NL63-infected cells ranged from being healthy to necrotic, with shedding of the dead and dying cells from the monolayer (Fig. 4). In the necrotic cells, virus was either free in the cytosol or present within vacuoles. Mature virions were seen “trapped” among the cilia in HAE cultures from 2 to 4 days after infection with HCoV-NL63. Collections of virions were seen at the bases of cilia, indicating that they likely had undergone exocytosis recently (Fig. 2C). These results are consistent with TEM analysis of SARS-CoV replication in HAE cultures (Sims et al., 2005).

3.3. Whole transcriptome amplification

For virus discovery efforts a random amplification approach was evaluated to determine whether viral nucleic acids could be detected from apical washes collected from infected cells. Apical washes from cells at 48 and 72 h post-infection were collected, cell debris was removed, and RNA was isolated from the medium. The RNA was subjected to reverse-transcription and amplification using random primers tagged with amplicon sequences as described in Section 2. This approach, termed whole transcriptome amplification (WTA), has been shown to generate an unbiased array of the sequences present in the sample, and has been used for amplification of quasispecies from unpurified influenza virus (Afonso, 2007; Nagy et al., 2005). WTA products generated from RNA isolated from the supernatants of mock-infected or HCoV-NL63-infected cells were analyzed by electrophoresis on a 1.5%

agarose gel (Fig. 5A), which revealed that abundant amplification products were generated from the HCoV-NL63-infected sample. The WTA products were cloned into the TOPO-TA cloning vector, transformed into *E. coli* and plasmid DNA was isolated and subjected to restriction enzyme digestion to determine the size of inserted sequences. Inserts ranged in size from approximately 50 to 500 nucleotides (Fig. 5B), and plasmid DNAs with inserted sequences were subjected to DNA sequencing and NCBI-BLAST analysis. Sequence analysis of randomly isolated clones revealed that 1 out of 63 (1.6%) was of viral origin (Fig. 5C). Similar to results obtained with WTA, high throughput pyrosequencing of HCoV-NL63 inoculated HAE cultures revealed that approximately 1% of clones were HCoV-NL63 (data not shown). The remaining clones which were identified by BLASTN or TBLASTX analysis represented a variety of human DNA sequences. Subsequent studies indicate that *DNase I* treatment after RNA isolation reduced cloning of chromosomal DNA sequences although a background of human ribosomal RNA and mRNAs was still detected. Overall, WTA is an effective method for random amplification and detection of viral sequences from supernatant of HAE cultures experiencing low-level viral replication, in a background of cellular sequences.

4. Discussion

4.1. Toward the identification of novel respiratory viruses

Given the uncertainty in choosing an optimal cell line for propagating unknown viral agents, the use of HAE cultures provides an unrivaled environment for the culturing of human respiratory viruses. Furthermore, limitations such as the lack of antibody reagents and minimal cytopathic effect can be circumvented

through the use of alternative methods such as transmission electron microscopy and whole transcriptome amplification to identify human respiratory viruses, as we have demonstrated using the model virus, HCoV-NL63. Establishing and validating this method will allow for future screening of acute clinical respiratory secretions from patients suffering from illnesses of unknown etiology. Although HAE cultures are labor intensive to establish, they are a valuable research tool for analysis of human respiratory pathogens. To evaluate clinical respiratory samples the respiratory secretions must be filtered to remove bacteria prior to inoculation of HAE cultures. Preliminary studies using application of filtered clinical samples to HAE cultures have not revealed detrimental effects to the HAE cultures.

The development of an accurate and rapid method to identify unknown respiratory pathogens is greatly needed, because previous studies have shown that up to 39% of respiratory tract infections have no identifiable causative agent (Iwane et al., 2004; Jartti et al., 2004). This approach may be useful in investigating the etiology of Kawasaki disease, a potentially fatal childhood illness of unknown etiology that is the leading cause of acquired heart disease in children in developed nations. Epidemiologic, immunologic and pathologic data suggest that a pathogen replicating in the respiratory tract is likely responsible for Kawasaki disease (Rowley et al., 2008). The methods described here may be useful for the identification of elusive human respiratory viruses.

This study represents a hybrid of cell-based and molecular approaches for human respiratory virus detection. HAE cultures were exploited for propagation of a human respiratory virus, HCoV-NL63, and viral replication was detected using an immunofluorescence assay, TEM analysis and a sequence-independent amplification approach. WTA analysis of HCoV-NL63 inoculated HAE cultures revealed that 1.6% of clones were HCoV-NL63 positive. In comparison, Palacios et al. (2008) identified viral sequences in 14 out of 103,623 (0.01%) randomly amplified cDNAs generated from a patient who succumbed to an unknown viral illness. Thus, the ability to amplify and isolate virus from supernatants has the advantage of reducing the background of human cellular sequences.

The successful use of HAE culture in this model system allowed for the first visualization of HCoV-NL63 by TEM, which revealed that HCoV-NL63 has the morphologic features of classic human and non-human coronaviruses: 75–110 nm spherical envelope, 10–20 nm club-shaped spikes, and virion assembly by budding into smooth-walled vesicles. TEM is a powerful tool for the rapid identification of unknown human respiratory viral pathogens as demonstrated by its use in the initial identification of SARS as a member of the *Coronaviridae* family (Ksiazek et al., 2003). In summary, experimental demonstration of the use of HAE cultures for the propagation and TEM visualization of human respiratory virus pathogens and sequence-independent amplification of nucleic acid isolated from the supernatant of the infected cultures to identify specific sequence of the virus have been demonstrated.

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