DETECTION OF HUMAN METAPNEUMOVIRUS INFECTION IN CHILDREN AND ADULTS BY MOLECULAR BASED METHODS

by

Ryan Dare

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This Thesis was presented

by

Ryan Dare

It was defended on

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and approved by

Thesis Advisor:
Charles Rinaldo, PhD
Chairman and Professor
Assistant Director of Clinical Microbiology
Department of Pathology, School of Medicine
Department of Infectious Diseases and Microbiology, Graduate School of Public Health
University of Pittsburgh

Committee Member:
Frank Jenkins, PhD
Associate Professor
Department of Pathology, School of Medicine
Department of Infectious Diseases and Microbiology, Graduate School of Public Health
University of Pittsburgh

Committee Member:
Clareann Bunker, PhD MPH
Assistant Professor
Department of Epidemiology
Graduate School of Public Health
University of Pittsburgh

Charles Rinaldo, PhD

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Ryan Dare, MS

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Human metapneumovirus (hMPV) is a recently discovered paramyxovirus known to cause respiratory tract infections primarily in children. This previously unknown pathogen remained undetected for years due to very slow replication *in vitro* and an inconsistent CPE. More recently, detection of hMPV by means of quantitative molecular techniques has proved to be more effective than culture methods. In this study we describe the development of a quantitative real time RT-PCR assay targeting the hMPV nucleoprotein (N) gene. This assay is compared to a real time nucleic acid sequence based amplification (NASBA) test, developed by bioMérieux, using control material from hMPV strains Can97-83 and Can98-75 representative of the two main lineages A and B, respectively. Using control material the real time RT-PCR, designed to detect all four sublineages of hMPV, can detect as low as 50 and 100 copies of viral RNA from the A and B lineages respectively. The real time NASBA assay can also detect 50 copies of viral RNA from the A strain but only detects 1000 copies of strain B viral RNA. In this study, hMPV has been detected in both immunosuppressed lung transplant recipients (2.14%) and children with respiratory symptoms (1.83%). This research is of major public health significance due to the amount of respiratory infections that are going undiagnosed or being treated with unnecessary antibiotics. It is important for our physicians to not only know that hMPV is present in our community but also to be able to detect and treat it appropriately. This study reports the first evidence of hMPV in the Pittsburgh area and demonstrates the importance of this virus as a critical player among respiratory pathogens in both immunosuppressed lung transplant recipients

and children. In conclusion, we have successfully developed a real time RT-PCR assay targeting the hMPV N gene. Using this assay along with the real time NASBA assay developed by bioMérieux, we have detected hMPV infections in lung transplant recipients in a year long study. Using the real time RT-PCR assay alone hMPV has also been detected in children suspected of respiratory infection during the early winter season.

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1. Introduction

Human metapneumovirus (hMPV) was first described in 2001 as a novel paramyxovirus isolated from the respiratory tract of children in the Netherlands (1).

Since its initial description, hMPV has been reported all over the world (2-9) and detected in patients ranging in age from 2 months to 87 years old, though children appear to be the most affected population (10). Retrospective studies have shown that hMPV is responsible for 4.5-17% of acute respiratory tract infections (ARTIs) in children, making it the second most prevalent respiratory virus in this age group following only respiratory syncytial virus (RSV) (3,4,11,12). In addition, 6.2-39% of ARTIs negative for other common respiratory viruses are positive for hMPV (5-7,13-16) and 1.2-4.1% of asymptomatic individuals are positive for hMPV, indicating the possibility of unapparent infections (12,16). Population based studies looking at the serostatus of individuals show that the virus has been circulating in the population since at least 1958 and nearly all children are seropositive by the age of five (1).

Studies have closely associated hMPV and RSV infections due to their ability to affect the same patient population during the late winter season (Jan- Apr). ARTIs of viral origin are the number one cause of pediatric hospitalizations during the winter season. RSV is responsible for the majority of ARTIs, followed by hMPV, then parainfluenza virus (PIV)-3 with incidence rates of 20%, 6.2%, and 3.1% respectively (15). Like RSV, hMPV causes occasional upper respiratory tract infections, although lower respiratory tract infections resulting in bronchiolitis, pneumonitis, bronchitis, pneumonia, and asthma are more common. Bronchiolitis is the most common (24-86%) of these diagnoses from hMPV infections (4-6,11,16,17). The clinical signs and symptoms for RSV and hMPV in children are indistinguishable, however RSV infects at an

earlier age than hMPV as evidenced by a seroprevalence of 48% and 11% respectively in patients less than 1 year old (18). hMPV signs and symptoms are consistent with those of RSV including dyspnea (83.3-95%), fever (52-91.7%), cough (69-90%), rhinorrhea (69-77%), weezing (50-52%), hypoxemia (31%) and croup (18%) (5,11,12,16). Hoarseness occurs in 91% of hMPV infected individuals, making it the only symptom that is significantly different from RSV (42%) (12).

hMPV is known to infect primarily children, but has been seen in all ages of people throughout many geographical locations. Critical hMPV cases have been reported in elderly and immunosuppressed individuals, though there have yet to be prevalence studies in many of these various patient populations. Solid organ transplant recipients are extremely immunosuppressed leaving them susceptible to opportunistic infections. Lung transplantation is an option for patients with end-stage pulmonary vascular or parenchymal disease. Among the potential post transplant complications, obliterative bronchiolitis (OB) is the most significant. This disease affects 34% of lung transplant recipients and is accompanied by a high mortality rate resulting in 1/3 of all deaths one-year post transplant. OB is characterized by partial or complete luminal obliteration of the bronchioles causing obstruction of airflow though the exact pathogenesis is not well understood (37-39). Respiratory viral infections have been postulated to be associated with the development of OB since immunosuppression leaves lung transplant recipients more susceptible to community-acquired infections (40-44). RSV, a paramyxovirus, has been shown to cause the majority of viral infections in lung transplant recipients, however, there is evidence that other paramyxoviruses such as PIV and hMPV are likely to initiate both chronic and acute rejection (41). The prevalence of OB increases from 34% to 47% in lung transplant recipients during respiratory virus season indicating that a respiratory virus could be involved in disease

pathogenesis (42,45). Lung transplant recipients have therefore been examined in this study to determine the prevalence of hMPV infection in this patient population and to determine if hMPV infection is correlated with OB status.

hMPV is a negative sense single stranded RNA virus (13.3kb) that has been added to the pneumovirus subfamily and metapneumovirus genus due to its genetic relatedness to the avian pneumovirus (APV). hMPV is most similar to subtype APV-C, sharing an identical order of genes with 56%-88% homology at the nucleic acid level. This illustrates a closer linkage between APV-C and hMPV than APV-C and APV subtypes A, B, and D (19). Of all human viruses, hMPV is most similar to RSV differing only in the order of genes and the absence of two non-structural genes, NS1 and NS2, however, they are only 30% genetically homologous at the nucleic acid level (20) (Figure 1). hMPV has two main genetic lineages, A and B, with two subtypes for each lineage (A1, A2, B1, B2) (21-23). The genetic homology, at the sequence level, within each lineage is 77%-95% and between each lineage is 59%-87% with the N, F, L, and M genes being the most conserved and the G and SH genes being the least conserved (24). Both lineages circulate at the same time with no evidence of geographic clustering or antigenic drift (21), however different subgroups may circulate at various rates during different seasons (23). There are reports of a patient being infected with both lineages in less than one month (25). Infection of hamsters with Can97-83 (strain A2) yields sera that neutralize infection with Can98-75 (lineage B2) and vice versa. This proves that the two separate lineages are highly related antigenically (48%) and are not distinguished serologically. (26)

Genetic similarity of hMPV and RSV

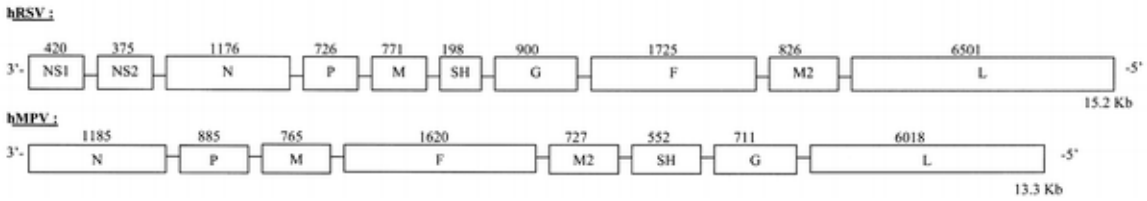


Figure 1: Genomic arrangement of RSV (top) and hMPV (bottom). RSV is the most analogous human virus to hMPV as evidenced by their genetic similarity. The only differences are the arrangement of genes and the presence of two nonstructural genes (NS1 and NS2) in RSV.

Viral infections can be detected in patient specimens by cell culture using a variety of continuous cell lines; respiratory viruses are usually detected from human and non human epithelial and kidney cell lines. These tests often require long incubation times, as virus-induced cytopathic effect (CPE) only becomes evident after many rounds of viral replication and spread of virus to nearby cells. The time needed to establish CPE depends on the virus and cell types, so results are often not available for 7-28 days after inoculation of the cell culture making it difficult to treat infected patients in a timely manner. Thus, the amount of time needed to detect virus in a sample can vary greatly. Although culturing virus does take a long time to yield results, it is considered the gold standard for viral detection.

A more rapid, commercially available, culture system using R-Mix shell vials detects respiratory viruses in less than 24 hours. This system contains a mixed monolayer of two cell lines, mink lung cells (Mv1Lu) and human adenocarcinoma cells (A549), that in combination support the detection of many viruses including influenza A and B, RSV, adenovirus, and PIV 1-3, however, hMPV is not detected by this method. An R mix vial is inoculated, cultured then stained with a pool of respiratory virus fluorescent antibodies. Positive screens are further identified with the second R-Mix shell vial that is stained with virus specific monoclonal antibodies for diagnosis. This type of testing will detect viral antigen prior to the production of CPE in an infected cell culture so is more rapid and very cost effective.

hMPV has gone unrecognized for many years because it displays very slow replication kinetics in vitro, does not replicate efficiently in continuous cell lines, and requires trypsin for growth (1). The virus can be cultured best in a rhesus monkey kidney cell line (LLC-MK2) (1,11,26,27) and studies have reported replication in HEp-2 and Vero cells (27-29), however it does not propagate in A-549, RD, 293, HT-29, and MDCK cells (11). Although hMPV can infect LLC-MK2 cells, CPE is not seen until an average of 17.3 days post infection (d.p.i) (11). This makes it very difficult to detect hMPV in a timely manner and optimize patient treatment.

Recent advancements in molecular technology have vastly improved diagnostic tests for infectious diseases. Nucleic acid amplification testing (NAAT) uses modern techniques such as polymerase chain reaction (PCR) and transcription assays to detect DNA or RNA from viral and bacterial pathogens. These assays are more sensitive than culture techniques and take only a few hours to detect the presence of the pathogen. Total nucleic acid can be extracted from patient specimens, which includes host DNA along with the presence of viral or bacterial nucleic acid in the case of infection.

PCR uses sequence specific primers and *Taq* polymerase to amplify target DNA by denaturing, annealing, and extending DNA sequences in a cyclic fashion. Reverse transcriptase-PCR (RT-PCR) has an initial RT step producing cDNA from RNA prior to amplification of the template. Both of these techniques amplify DNA (or cDNA) every cycle in a binary fashion, resulting in large quantities of amplicon which can then be detected by gel electrophoresis. In the case of real time PCR and real time RT-PCR, simultaneous amplification and detection occurs using molecular probes labeled with a fluorophore. New minor groove binding (MGB) molecular probes are smaller and claim to be more specific than original probes. Real time amplification has further improved clinical diagnostics by decreasing turn around time omitting

post amplification processing. In addition, real time amplification is a closed system greatly reducing the risk of contaminating other specimens or reagents. The real time PCR and RT-PCR systems are also quantitative, benefiting patient care in situations where the viral load is indicative of treatment.

Transcription assays, such as nucleic acid sequence based amplification (NASBA) and transcription mediated amplification (TMA), are alternative amplification systems to RT-PCR for RNA targets, however similar in theory. These assays are isothermal in comparison to the cyclic variation in temperature during PCR. NASBA uses three enzymes: AMV-RT, RNase H, and T7 RNA polymerase (Figure 2), while TMA uses only two, RT and T7 polymerase during amplification, though both produce RNA transcripts in a logarithmic fashion compared to the binary fashion of PCR. NASBA has been shown to be more sensitive than RT-PCR for detection of some RNA viruses such as Dengue and West Nile virus (32, 33).

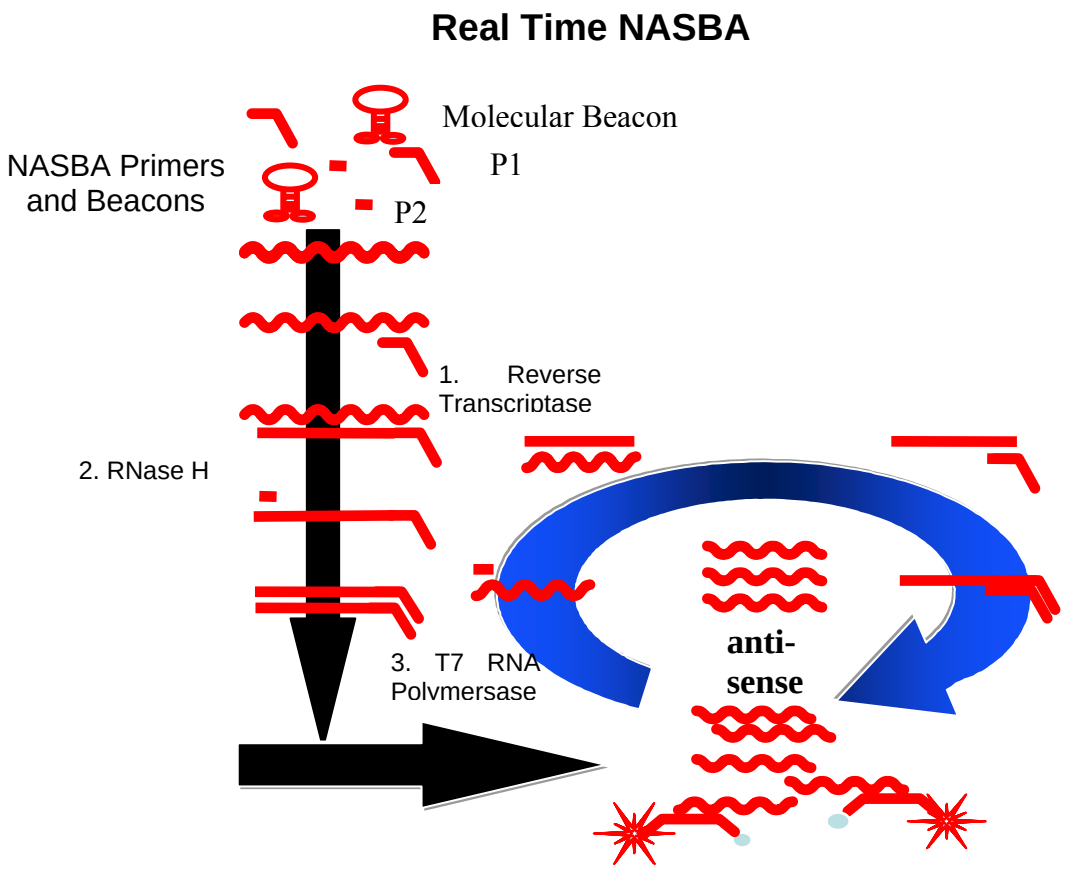


Figure 2: Nucleic acid sequence based amplification (NASBA). Isothermal amplification of RNA using three enzymes; AMV-RT, RNase H, and T7 RNA Polymerase. The RNA is reverse transcribed into cDNA which is then continuously transcribed back into RNA in a logarithmic fashion. RNase H degrades RNA from the RNA/DNA duplex following the first strand of cDNA synthesis. As the target is amplified, the molecular beacon emits fluorescence which is then detected by the EasyQ Analyzer.

Molecular tests in the clinical setting need appropriate positive, negative, and internal controls to assure that the tests are functioning correctly. When amplifying nucleic acid using platforms such as PCR or NASBA, extraction and amplification controls are both needed. This is to ensure that the entire process from beginning to end is working properly. Positive and negative controls measure the ability of the assay to detect positive material as positive and fail to detect negative material as positive to determine if the entire test is working as it should. Positive controls for viral testing are usually virus or viral nucleic acid containing the target sequences spiked into a same matrix to which the specimens are added. Negative controls would be extracted in matrix alone. Internal controls account for the integrity of each individual specimen indicating that

RNA from virus was extracted and amplified as it should have. This can be done by amplifying the RNA of "housekeeping" genes, such as ß-actin, which should be consistently present in certain specimens. An alternative is to spike a non-human DNA or RNA virus control into each specimen prior to extraction. This yields a consistent quantity of amplified internal control compared to a housekeeping gene that will vary between specimens. With a quantitative real time amplification system, this will show if there is any form of inhibition.

RT-PCR has become the preferred method for diagnosing hMPV infections due to a decrease in throughput time and better sensitivity to other diagnostic assays. The nucleoprotein (N) and polymerase (L) genes have been the most frequently utilized targets due to their conserved nature across hMPV strains (7,30,31). One of these assays has reported to detecting as low as 5-10 copies of hMPV viral RNA (31). There is, however, a lack of published real-time RT-PCR assays designed to specifically detect all four lineages of hMPV. Though bioMérieux has been developing a real time NASBA assay for hMPV, nothing has been published. The need to develop a real time RT-PCR that will detect all four sublineages of the virus is imperative because most of the published assays are designed to target the prototype A1 strain.

To design an optimal molecular assay for any infectious disease, a thorough genetic analysis of all strains of the pathogen must be considered prior to designing primers and probes for detection. The sequences of all lineages and sub-lineages must be aligned to find conserved regions among all strains. In addition, known single nucleotide polymorphisms (SNPs) should be noted such that primers are designed not to anneal where these mismatches frequently occur. The objective is to have an assay that will detect all positive samples from all genetic lineages of the virus or bacteria.

There is no specific treatment available for hMPV. Ribavirin and immune globulin designed for intravenous administration (IVIG) have broad antiviral activity and have inhibited this virus in vitro. However, both treatments are extremely expensive, Ribavirin is a teratogenic agent and IVIG can interfere with live virus vaccines (e.g. MMR and varicella) (32). A new sulfated sialyl lipid molecule, NMSO3, is known to have antiviral activity against RSV. NMSO3 also shows inhibition of hMPV infection in vitro but not against other paramyxoviruses such as PIV and measles. This agent diminishes syncytia formation and the secondary spread of infection. (33) Vaccines are not commercially available, though a recently described reverse genetic system (34) has been used to develop a recombinant hMPV virus lacking the attachment gene (G) (35). Hamsters inoculated with this rhMPVΔG virus experience a 40 fold decrease in viral replication compared to wt virus. In addition, high titers of hMPV neutralizing serum antibodies are produced resulting in complete protection against wt virus challenge. (35) This system represents a vaccine candidate with effective protection against hMPV to be studied further in primate models.

Human metapneumovirus is of great public health significance because it is an established community acquired respiratory virus that infects young children, elderly adults, and immunocompromised individuals causing bronchiolitis in the majority of cases. In the United States alone, bronchiolitis is responsible for over 150,000 hospitalizations annually (5). Fatal cases have been reported in which hMPV is the sole pathogen isolated from lung tissue post mortem (11,36). Due to its recent description, little is known about the severity of disease in specific patient populations. Development of a sensitive diagnostic test and analysis of patient specimens in various cohorts will be beneficial for further understanding of hMPV. In this study, an in-house real time RT-PCR assay specific for hMPV detection has been developed and

it is directly compared to a real time NASBA assay developed by bioMérieux. Bronchoalveolar lavage (BAL) specimens from lung transplant recipients and nasal swabs from symptomatic children were examined for hMPV infection by both systems. I hypothesize that detection of hMPV will occur in the lung transplant patient population resulting in severe infection causing a predisposition to the development of OB. In addition, hMPV will be detected in the pediatric population at rates lower than RSV but higher than PIV.

2. Specific Aims

I. Develop a specific and sensitive real time RT-PCR diagnostic assay for the detection of all four hMPV genetic lineages from respiratory specimens.

II. Produce hMPV RNA transcripts of the gene targeted in the diagnostic assay for positive material to be used for standard curves and positive controls during specimen testing.

III. Using the recently developed real time RT-PCR and previously developed real time Nucleic Acid Sequence Based Amplification (NASBA) diagnostic assays, test patient respiratory specimens for the presence of hMPV viral RNA in two separate cohorts:

    a. Bronchoalveolar lavage (BAL) specimens from immunosuppressed lung transplant recipients of all ages at University of Pittsburgh Medical Center (UPMC).

    b. Nasopharyngeal (NP) swabs from symptomatic children tested for *Bordetella pertussis* at Children's Hospital of Pittsburgh (CHP).

IV. Test patient respiratory specimens from both cohorts for other respiratory viruses (Adenovirus, Influenza A and B virus, RSV, PIV1-3) by R mix at the Clinical Virology Laboratory (CVL) at UPMC.

3. Methods

3.1. Sample Collection:

BAL specimens were collected from adult lung transplant recipients. Bronchoscopies with bronchoalveolar lavage were performed at regular intervals according to the transplant protocol (1, 3, 6, 9, 12 months post-transplant) and as indicated by symptomatic events such as fever, radiographic infiltrates, and decreased forced expiratory flow as determined by spirometry. 100ul of BAL specimen was stored in lysis buffer (bioMérieux, Durham, NC) at -80ºC in a total volume of 1ml.

Nasopharyngeal (NP) swabs from children with respiratory symptoms suspected of *Bordetella pertussis* were collected using the Childrens Hospital of Pittsburgh (CHP) specimen collection kit and instructions. These specimens were collected during the 2004-2005 respiratory season from September to February. An NP swab composed of Dacron fiber tip was inserted past the posterior nasopharynx, held in place for 15-30 seconds or until the patient coughed. The tube was then placed in a tube of saline and transported to the lab where the swab was vortexed and wrung out such that the suspension can then be aliquoted (100ul) and stored at -80ºC until testing.

3.2. Virus and Viral RNA:

Viral isolates of hMPV strains Can97-83 (strain A2) and Can98-75 (strain B2) were kindly donated by Dr. Dean Erdman at the CDC (Atlanta, GA) and stored in TRIzol at -80ºC. Both strains were cultured in MK2 cells and virus was taken at the third passage in June of 2003.

Can97-83 viral RNA (761ng/ul) was kindly provided by Dr. Ursula Buchholz at the NIH (Bethesda, MD). Total RNA (840ng/ul) from infected vero cells and total RNA from uninfected

vero cells were taken 72 hrs post infection from 25cm^2 flasks. Total RNA was extracted using the RNeasy kit (Qiagen, Alameda, CA) in June of 2004.

Equine arteritis virus (EAV) was purchased from ATCC® (ATCC® Number: VR-796™) to be used as an internal control for RT-PCR of all respiratory specimens. The virus was inoculated into a 75cm^2 flask of vero cells near confluency. Infection resulted in detachment of the monolayer from the flask in less than 24 hours. The virus was harvested from the supernatant following sonication and stored (500ul:500ul) in minimal essential medium with 10% fetal bovine serum at -80ºC. A 1/50 dilution of the virus was spiked into each specimen prior to extraction.

3.3. Nucleic Acid Extraction:

Isolation of viral nucleic acid from control material and patient specimens were performed using a NucliSens® automated extractor (bioMérieux, Durham, NC) according to the manufacturer's instructions. Lysis of 100ul of sample in 900ul of lysis buffer (bioMérieux, Durham, NC) occurs for 30 minutes and is brought to a total volume of 1ml. Following lysis of the specimen, the internal control virus and 1ml of silica (53ul:90ul of silica to lysis buffer) was added. The solution was transferred into a closed system cartridge and placed onto the instrument for extraction. The procedure takes approximately 1 hour and yields 50ul of eluate which can then be stored directly at -80ºC in the same tube.

3.4. Primer Design:

hMPV specific primers and MGB probes were designed for optimal detection of all four hMPV genetic lineages by real time RT-PCR targeting the N, F, and L genes (Figure 3).

Genbank accession numbers for all four lineages are AY355324, AF371337, AY355335, and AY355328 for the N gene, AY304360, AF371337, AY304362, and AY304361 for the F gene, and AF371337, AY525843, AY297749 and AY297748 for the L gene. The primers and MGB probes were designed using Primer Express® software and the recommended primer/probe target regions were analyzed for variability among the four strains using Vector NTI 9.0. Primer/probe targets were selected based on the highly conserved regions using degenerate bases where necessary. All designed probes are 6-carboxy-fluorescein (FAM) labeled with an MGB quencher. The Maertzdorf primer/probe set, designated as N1, uses a TAMRA quencher and targets the 3` end of the N gene. The sequences of each primer/probe target are listed in Table 1.

Target sites on hMPV genome

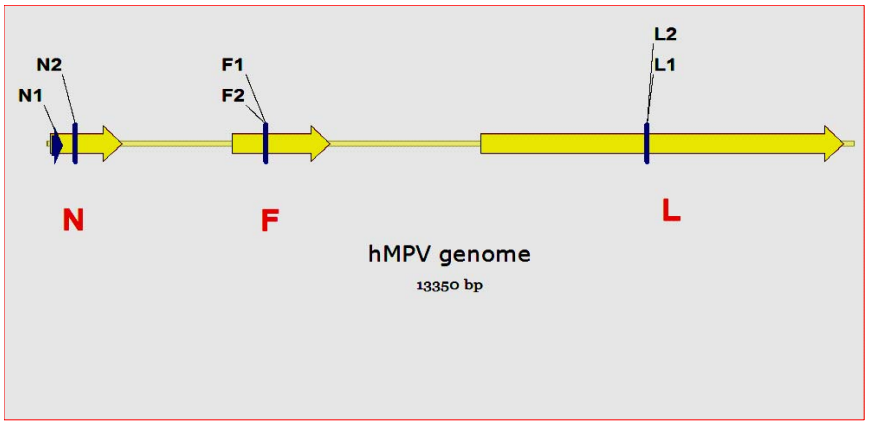


Figure 3: hMPV genome with N, F and L genes highlighted indicating location of each primer/probe set. N1 is the Maertzdorf et al (31) set and N2 is the designed MGB set.

Table 1: Primers and Probes

Target	Primer	Sequence
N	Forward	5`-CATCAGGTAATATCCCACAAAATCAG-3`
	Reverse	5`-GTGAATATTAAGGCACCTACACATAATAARA-3`
	Probe	5`-FAM-TCAGCACCAGACACAC-MGB-3`
F	Forward 1	5`-ARATGGCCGTTAGCTTCAGTCA-3`
	Forward 2	5`-AATGGCCGTTAGCTTCAGTCA-3`
	Reverse	5`-GTCTGAAAAYTGCCGCACAA-3`
	Probe	5`-FAM-TTCAACAGAAGGTTCCTAAAT-MGB-3`
L	Forward	5`-GGAGGAGGAGATCCAGTAGTYTTYT-3`
	Reverse 1	5`-CCACATGACTGATTGCTTCAGTTAA-3`
	Reverse 2	5`-TCCACATGACTGATTGCTTCAGT-3`
	Probe	5`-FAM-AGAAGGACCCCTGATTT-MGB-3`
Maertzdorf primers	Forward	5'-CATATAAGCATGCTATATTAAAAGAGTCTC-3
	Reverse	5'-CCTATTTCTGCAGCATATTTGTAATCAG-3'
	Probe	5'-FAM-TGYAATGATGAGGGTGTCACTGCGGTTG-TAMRA-3'
Cloning primers	Forward	5`-GAAAATGTCTCTTCAAGGGATTCAC-3
	Reverse	5`-GCCAATTTTGCCGCTTCAT-3`
	RT GSP	5`-GCCTTGCGAGACATTATGATTTG-3`
EAV	Forward	5`-GGCGACAGCCTACAAGCTACA-3`
	Reverse	5`-CGGCATCTGCAGTGAGTGA-3`
	Probe	5`-FAM-TTGCGGACCCGCATCTGACCAA-TAMRA-3`

Table 1: List of primers and probes for hMPV real time RT-PCR detection, production of an N gene amplicon by conventional PCR, and equine arteritis virus (EAV) internal control amplification by real time RT-PCR. The Y represents a pyrimidine (C or T) and the R represents a purine (A or G).

3.5. Real Time RT-PCR:

Reverse transcription and amplification of hMPV RNA was performed in a single tube using the in house developed N gene primers and probe, designated as N2 above, and One step RT qPCR MasterMix (Eurogentec, San Diego, CA) according to the manufacturer's instructions. This 25ul reaction consisted of 2x reaction buffer, 8mM $MgCl_2$, 200x EuroScript RT 0.25U/µl &

RNase Inhibitor 0.1u/ul, 300nM forward primer, 900nM reverse primer, 100nM probe, template RNA, and sterile H_2O. The amplification was carried out using an ABI PRISM® 7000 (Applied Biosystems, Foster City, CA) with the following cycling conditions: [RT step 48º C, 30 min]; [Taq Activation step 95º C, 10 min] x 1 followed by [PCR step 95º C, 15 sec; 60º C, 1 min] x 45.

Optimization of other hMPV assays were carried out under the same conditions however, primer, probe, and $MgCl_2$ concentrations varied (Table 2). The F1 assay consisted of 300nM of primers, 200nM of probe, and 8mM of $MgCl_2$. The F2 assay used 300nM of forward primer, 900nM of reverse primer, 250nM of probe, and 6mM of $MgCl_2$. The L1 assay consisted of 100nM of forward primer and probe, 900nM of reverse primer, and 5mM of $MgCl_2$. The L2 target used 900nM of primers, 100nM of probe, and 6mM of $MgCl_2$. A previously published hMPV primer/probe set (Maertzdorf et al) targeting the 5`end of the N gene was used as a reference assay (Table 1). The Maertzdorf set consisted of 900nM primers, 100nM of probe, and 5mM of $MgCl_2$.

Amplification of EAV served as an internal control in every patient specimen to determine the integrity of the sample controlling for both extraction and amplification procedures. The assay conditions for EAV amplification are the same as they are for hMPV described above, however, 800nM of primers and 320nM of the probe were used to detect EAV (Table 1).

3.6. Real Time NASBA:

Real time NASBA was performed using NucliSens Basic Kit reagents (bioMérieux) on an EasyQ Analyzer (bioMérieux, Durham, NC) for 120 minutes according to the manufacturer's instructions. Working in collaboration with bioMérieux, hMPV specific primer/beacon mix was provided for virus amplification and detection. Each sample was run in a 20-µl NASBA reaction

mixture containing; 5ul of template, 5ul of enzyme (1 enzyme sphere diluted in 55ul of enzyme diluent), and 10ul of reagent mastermix (1 reagent sphere, 80ul of reagent diluent, 16ul of 80mM KCl, 10ul of primer/beacon mix, and 14ul of H_2O). Template was added to the reagent mastermix and incubated at 65°for 2 min and 41° for 2 min. Following the incubation, enzyme was added and mixed by briefly spinning and flicking the tubes. Once sufficient mixing had occurred the specimens were loaded onto the EasyQ analyzer for amplification.

3.7. Viral Culture and Immunofluorescent Staining:

Two R mix shell vials (35-37°C) were inoculated with 200ul of clinical specimen according to the manufacturer's instructions. Each vial was centrifuged at 700 x *g* for 60 min and incubated at 35°C. Screening of the R-Mix shell vial at 18 to 24 h postinoculation was accomplished with the first of the two vials. Coverslips were fixed with acetone and stained with a pool of respiratory virus fluorescent antibodies (adenovirus, influenza A and B virus, RSV and PIV 1-3) (Bartels, Inc, Issaquah, WA) according to the manufacturer's instructions. Positive specimens were further identified with the second R-Mix shell vial by scraping and spotting eight-well slides. These slides were fixed with acetone again and stained with virus-specific monoclonal antibodies (Bartels, Inc, Issaquah, WA). If the initial R-Mix screen was negative, the second vial was discarded.

3.8. Construction of N Gene Plasmid:

The hMPV N gene, in its entirety, was amplified by conventional two step RT-PCR from the Can97-83 viral RNA. Primers were designed using Primer Express® software 2.0 looking at the first 1350 bases of the Can97-83 genome. A gene specific primer (GSP) downstream of the N

gene located in the F gene region was designed for reverse transcription 900nM (5`-GCCTTGCGAGACATTATGATTTG-3`). The forward primer is upstream of the N gene start site (5`-GAAAATGTCTCTTCAAGGGATTCAC-3`) and the reverse primer is downstream of the N gene stop codon (5`-GCCAATTTTGCCGCTTCAT-3`). Both primers were used at 300nM concentration. First strand cDNA synthesis was generated using Superscript III RT (Invitrogen, Carlsbad, CA). A 10ul reaction consisting of 10ug of RNA, 2uM of primer, 10mM dNTP mix, and H_2O is incubated at 65°C for 5 minutes. Following incubation, 10x RT buffer, 25mM $MgCl_2$, 0.1 M DTT, RNaseOUT (40U/ul), and Superscript III RT (200U/ul) was added sequentially in a 2:4:2:1:1 ratio, respectively. Reaction was incubated at 50°C for 50 minutes. Amplification of the N gene cDNA was done using a FailSafe™ PCR system (EPICENTRE® Biotechnologies, Madison, WI). The failsafe premix D, containing dNTPs, buffer, and various amounts of $MgCl_2$ and FailSafe™ PCR Enhancer with Betaine, was used for optimal amplification. Amplification parameters were [98º C, 2 min hold; followed by 35 cycle of 98º C, 20 sec, 54º C, 30 sec, 68º C, 1 min]. Presence of the amplicon was analyzed on a 1% agarose gel in Tris-EDTA (TE) buffer.

The PCR product was gel purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI) according to the manufacturer's instructions and ligated into the pGEM®-T Easy Vector (Promega, Madison, WI) overnight at 4ºC according to the manufacturer's instructions. The construct was then transformed into competent JM109 *E. coli* cells by heat shocking them at 42ºC for 45-50 seconds. Cells were plated on LB/Amp/IPTG/X-gal plates and screened for effective transformation by blue/white selection. White colonies were cultured overnight in 3ml of ampicillin LB medium. Plasmid DNA was extracted using the

Wizard® Plus SV Miniprep DNA purification System according to the manufacturer's instructions (Promega, Madison, WI).

Insertion of the correct sequences into the plasmid was confirmed by digesting the plasmid with Pst I (Promega, Madison, WI) at 37ºC for 3 hours. Orientation of the insert was confirmed by a double digest with both Pst I and Hind III (Promega, Madison, WI) at 37ºC for 3 hours. Digests consisted of 2ul of a 10x reaction buffer, 0.2ul of BSA, 5ul of DNA, and H_2O to a volume of 20ul and were analyzed on a 1% agarose gel in TE buffer.

Plasmid templates were digested with Nco I (New England Biolabs, Inc, Beverly, MA) at 37ºC for 2 hours. After complete digestion, the restriction enzymes were heat inactivated at 65ºC for 20 minutes and the DNA from the digest was isolated using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI) according to the manufacturer's instructions.

Under control of the SP6 promoter, the plasmid produced RNA run off transcripts of the entire N gene using 7.625ug of RNA and the Ribomax™ Large Scale RNA Production System (Promega, Madison, WI) according to the manufacturer's instructions. Following the transcription assay, the sample was DNase treated and RNA purified by phenol chloroform extraction. The hMPV N gene transcripts were quantified using a spectrophotometer to measure the *A*260 yielding 4.563×10^{12} copies/ul. A 1/300 dilution of the transcripts gave an A260 of 0.256. 40ug/ml x 0.256 x 300=3.072ug/ul. 330Daltons x 1271 RNA bases = 419,430 molecular weight. .000003072g / 419430 = 7.324×10^{12}. 6.23×10^{23} x7.324×10^{-12} = 4.563×10^{12}.

4. Results

4.1. Primer Design:

Three conserved genes (N, F and L) were selected as potential target sites to detect every hMPV strain from all known genetic lineages by real time RT-PCR. One primer/probe set was designed for the N gene; however, a published set for the same gene was also analyzed (31). Two primer/probe sets were designed for the F gene using the same molecular beacon and reverse primer but with a different forward primer. Two primer/probe sets were also designed for the L gene using the same molecular beacon and forward primer however a different reverse primer was used. Each primer and probe was designed such that there were minimal mismatches among the four strains. Degenerate nucleotides were used in positions where bases varied between strains in order to enhance binding and amplification when applicable. Every position containing a mismatch could not use degenerate bases due to the addition of an alternate base shifting the Tm out of range for appropriate PCR conditions. Table 3 shows the alignment of each primer and probe with the corresponding sequences from each of the four strains. The hMPV prototype strain NL/01/00 was used to analyze the A1 sequences. A2 sequences were extracted from the NL/17/00 strain for N and F gene analysis and Can97-83 for L gene analysis. The NL/99/01 strain was used as a reference for B1 sequence analysis for all three genes. The B2 sequences for the N and L genes were extracted from the NL/94/01 strain however the Can98-75 strain was used for the L gene analysis. Each primer and probe is specific to metapneumovirus as evidenced by performing a blast search against all other known genetic sequences (data not shown).

Table 2: Primer and probe alignment with corresponding sequences from all four hMPV genetic lineages

Maertzdorf et al	Forward	Reverse	Probe
Primer Sequence	CATATAAGCATGCTATATTAAAAGAGTCTC	CCTATTTCTGCAGCATATTTGTAATCAG	TGYAATGATGAGGGTGTCACTGCGGTTG
A1	CATACAAGCATGCTATATTAAAAGAGTCTC	CCTATTTCTGCAGCATATTTGTAGTCAG	TGYAATGATGAGGGTGTCACTGCGATTG
A2	CATACAAGCATGCTATATTAAAAGAGTCTC	CCTATTTCTGCAGCATATTTGTAATCAG	TGYAATGATGAGGGTGTCACTGCGGTTG
B1	CATATAAGAATGCTATATTAAAAGAGTCTC	CCTATCTCAGCAGCATATTTGTAATCAG	TGYAATGATGAAGGTGTCACTGCGATTG
B2	CATATAAGAATGCTATATTAAAAGAGTCTC	CCTATCTCTGCAGCATATTTGTAATCAG	TGYAATGATGAAGGTGTCACTGCGATTG

N gene	Forward	Reverse	Probe
Primer Sequence	CATCAGGTAATATCCCACAAAATCAG	GTGAATATTAAGGCACCTACACATAATAARA	TCAGCACCAGACACAC
A1	CATCAGGTAATATCCCACAAAATCAG	GTGAATATTAAGGCACCTACACATAATAAGA	TCAGCACCAGACACAC
A2	CATCAGGCAATATTCCACAAAATCAG	GTAAATATTAAGGCACCTACACATAATAAGA	TCAGCACCAGACACAC
B1	CATCAGGTAACATCCCACAAAACCAG	GTGAATATTAGGGCACCTACACATAATAAAA	TCAGCACCAGACACAC
B2	CATCAGGCAACATCCCACAAAACCAG	GTGAATATTAAAGCACCTACACACAATAAAA	TCAGCACCAGACACAC

F gene	Forward 1	Forward 2	Reverse	Probe
Primer Sequence	ARATGGCCGTTAGCTTCAGTCA	AATGGCCGTTAGCTTCAGTCA	GTCTGAAAAYTGCCGCACAA	TTCAACAGAAGGTTCCTAAAT
A1	AAATGGCCGTTAGCTTCAGTCA	AATGGCCGTTAGCTTCAGTCA	GTCTGAAAATTGCCGCACAA	TTCAACAGAAGGTTCCTAAAT
A2	AAATGGCTGTTAGCTTCAGTCA	AATGGCTGTTAGCTTCAGTCA	GTCTGAAAATTGCCGCACAA	TTCAACAGAAGGTTTCTAAAT
B1	AGATGGCTGTCAGCTTCAGTCA	GATGGCTGTCAGCTTCAGTCA	GTCTGAAAACTGCCGCACAA	TTCAACAGAAGATTTCTAAAT
B2	AGATGGCTGTCAGCTTCAGTCA	GATGGCTGTCAGCTTCAGTCA	GTCTGAAAACTGCCGCACAA	TTCAACAGAAGATTCCTAAAT

L gene	Forward	Reverse 1	Reverse 2	Probe
Primer Sequence	GGAGGAGGAGATCCAGTAGTYTTYT	CCACATGACTGATTGCTTCAGTTAA	TCCACATGACTGATTGCTTCAGT	AGAAGGACCCCTGATTT
A1	GGAGGAGGAGATCCAGTAGTCTTCT	CCACATGACTGATTGCTTCAGTTAA	TCCACATGACTGATTGCTTCAGT	AGAAGGACCCCTGATTT
A2	GGAGGAGGAGATCCAGTAGTCTTCT	CTACATGGCTGATTGCCTCAGTTAA	TCTACATGGCTGATTGCCTCAGT	AGAAGGACCCCTGATTT
B1	GGAGGGGGAGATCCAGTAGTTTTTT	CCACATGGCTGAGGGCTTCAGTTAG	TCCACATGGCTGATTGCTTCAGT	AGAAGGACTCCCGATTT
B2	GGAGGAGGAGACCCAGTAGTCTTCT	CCACATGACTGATTGCTTCAGTTAA	TCCACATGGCTGATTGCTTCAGT	AGAAGGACTCCTGATTT

Table 2: Alignment of primer/probe sets with corresponding sequences from each of the four hMPV genetic lineages. The red bases indicate a mismatch and the blue bases indicate the use of a degenerate nucleotide. The Y represents a pyrimidine (C or T) and the R represents a purine (A or G). The Maertzdorf et al primer/probe set in addition to the designed N, F, and L primer/probe sets are included in the table.

4.2. hMPV Real Time RT-PCR Optimization:

Each of the six real time RT-PCR assays were optimized using a high concentration (20ng) of Can97-83 (strain A2) hMPV viral RNA. Cross titrations of primers ranging from 100-900nM final concentration and probes ranging from 100-250nM final concentration were performed in

addition to an $MgCl_2$ titration ranging from 5-10mM. The optimal concentrations for each assay and their respective Ct values are listed in Table 3.

Table 3: Optimal conditions for each hMPV real time RT-PCR assay

Target	Forward Primer (nM)	Reverse Primer (nM)	Probe (nM)	$MgCl_2$ (mM)	Ct Value
Maertzdorf	900	900	100	5	17.58
N gene	300	900	100	8	21.11
F gene (1)	300	300	200	8	19.81
F gene (2)	300	900	250	6	20.83
L gene (1)	100	900	100	5	24.76
L gene (2)	900	900	100	6	25.08

Table 3: Optimal conditions of each target using 20ng of hMPV Can97-83 (Strain A2) RNA. Mean Ct values are representative of duplicate samples

hMPV virus Can97-83 (strain A2) and Can98-75 (strain B2) were used to determine the capability of each assay to detect both main lineages of the virus (Table 4). This is a lower dilution than the amount used during the original optimization. This dilution was used in order to detect a concentration of virus that would more likely be seen in patient specimens. Most of the assays detected hMPV with similar efficiency during the initial optimization using RNA from strain A2. The F and L targets failed to detect RNA from strain B2 with comparable efficiency to strain A2. The Maertzdorf set detects high quantities of strain A2 very well; however, when both strains of the virus are diluted the ability of the primer/probe set to amplify virus appears to be abolished. This is due to amplifying hMPV under different conditions than used by the Maertzdorf lab. We can not alter our conditions and parameters due to maintaining a consistent platform to amplify many targets at once. The N gene target has the ability to detect both main lineages without a significant decrease in the limit of detection.

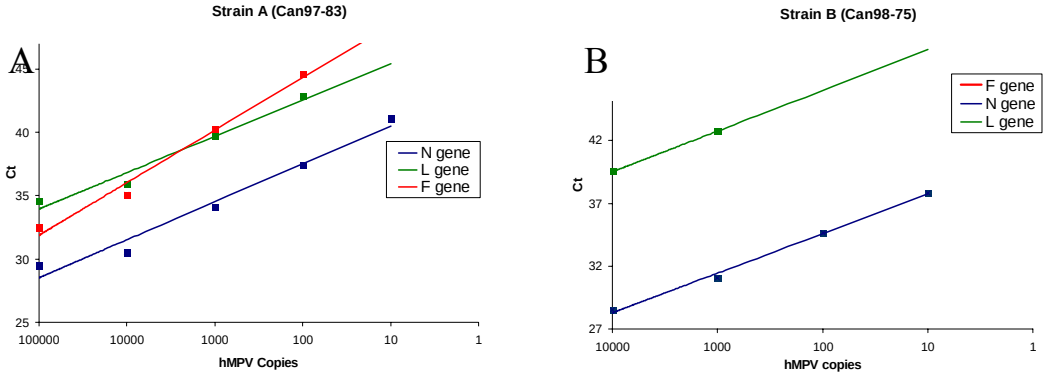
Table 4: Detection of hMPV strain A and B by real time RT-PCR

Target	Can97-83 (Ct)	Can98-75 (Ct)
Maertzdorf	Undet	Undet
N	29.77±0.36	31.16±1.31
F1	29.97±1.09	35.97±0.42
F2	29.52±0.87	35.73±0.42
L1	33.57±0.93	37.80±1.17
L2	34.33±1.15	33.09±1.81

Table 4: Sensitivity of each target amplifying a 1/1000 dilution of CDC hMPV RNA extracted in lysis buffer from strains Can97-83 (A2) and Can98-75 (B2). Mean Threshold cycle (Ct) values are representative of duplicate samples from two separate experiments.

Both strains of hMPV were quantitated by real time RT-PCR using RNA transcripts and the N gene primers. The superior of the two primer/probe sets for each gene was used to determine the lower limits of detection. The limit of detection for each gene was determined by amplifying a serial dilution of hMPV strains A and B ranging from 100,000-10 copies. The lower Ct values using the N target for detection indicate an impressive limit of detection for both main lineages of the virus as shown in figure 4A-B. The limit of detection for each target is shown in figure 4C. The N target detected 100 copies of both strains A and B, the F target only detected 1,000 copies of strain A and failed to detect 100,000 copies of strain B, and the L target detected 100 and 10,000 copies of strain A and B respectively.

Limits of Detection for hMPV strains A and B using real time RT-PCR



C

Target	Strain	100,000 copies	10,000 copies	1,000 copies	100 copies	10 copies
N gene	Can97-83 (A2)	+	+	+	+	-
	Can98-75 (B2)	+	+	+	+	-
F gene	Can97-83 (A2)	+	+	+	-	-
	Can98-75 (B2)	-	-	-	-	-
L gene	Can97-83 (A2)	+	+	+	+	-
	Can98-75 (B2)	+	+	-	-	-

Figure 4: Detection of serially diluted hMPV RNA from strains A(top left panel) and B (top right panel) by N (blue), F (red), and L (green) targets by real time RT-PCR. Limit of detection for each target.

Detection of hMPV in nucleic acid from clinical specimens by real time RT-PCR utilizing the N target was validated with hMPV negative BAL fluid from lung transplant recipients. Serial dilutions of hMPV RNA were spiked into 100ul of clinical specimens prior to extraction. As a control, the same dilutions of viral RNA were spiked into lysis buffer without specimen and extracted alone. Figure 5 shows the detection of hMPV under both of these conditions in addition to amplified RNA from the same dilutions without an extraction procedure. Various quantities BAL fluid ranging from 10ul to 100ul were extracted and amplified to see if any inhibitory effect occurred. There was no inhibition noted up to 100ul of specimen tested (data not shown). Proteinase K treatment of BAL specimens prior to addition of the lysis buffer did

not show any advantage for viral detection (data not shown). These data confirm that hMPV RNA can be detected in clinical patient specimens.

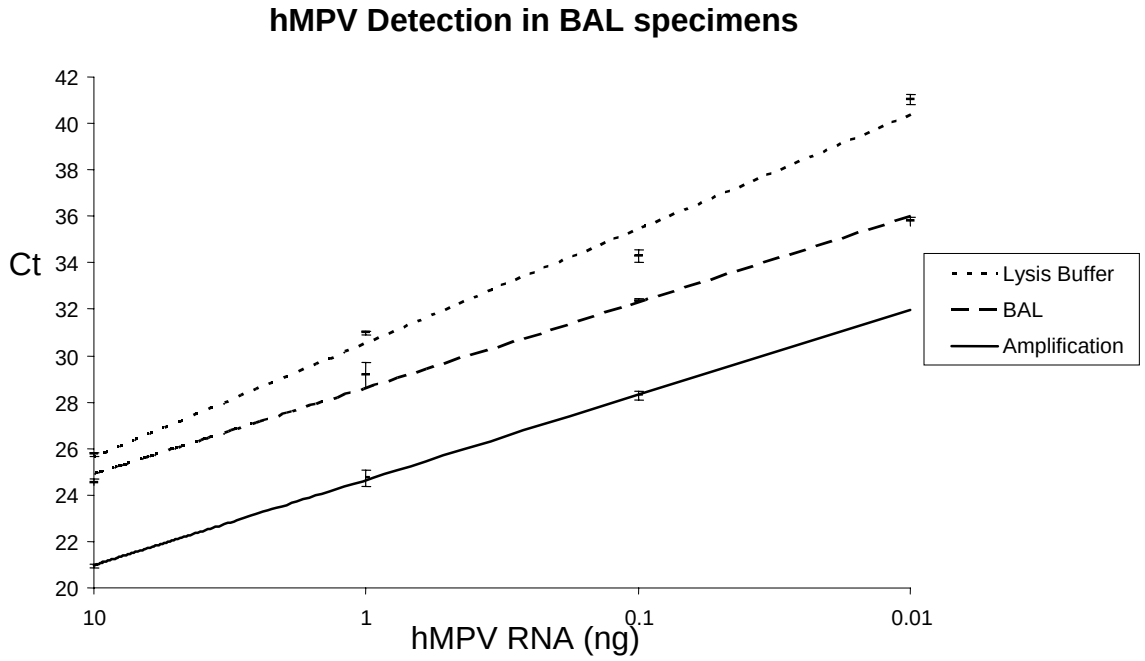


Figure 5: Detection of Can97-83 hMPV RNA by real-time RT-PCR. 10, 1.0, 0.1, and 0.01ng of hMPV RNA was spiked into either hMPV negative BAL specimens (-----) or Lysis Buffer alone (- - -) followed by extraction of total nucleic acid. Amplification of extracted RNA was performed in duplicate using the Nucleoprotein (N) target. Equivalent amounts of amplified (non-extracted) viral RNA are shown by (-) to compare with extracted RNA.

Equine arteritis virus (EAV), an RNA virus, was used as an internal control to monitor extraction and amplification of all patient specimens. A 1:50 dilution of cultured virus was spiked into each specimen prior to extraction. Specimens are considered inhibited and must be repeated if the EAV Ct value is >30.77. This was determined by taking the average Ct value+2SD of 80 BAL specimens. Detection of the internal control by real time RT-PCR using published target sequences was optimized for the ABI PRISM® 7000 on our platform. The EAV caused no inhibition of hMPV amplification even at low levels of spiked hMPV (Table 5)

Table 5: EAV optimization as an internal extraction and amplification control

hMPV load	10ng	1ng	100pg	10pg	1pg	0.1pg
HMPV alone	22.3±0.34	26.9±0.49	30.6±0.21	33.1±0.13	40.8±2.19	40.2±0.18
HMPV with EAV	21.5±0.04	27.7±0.19	30.0±0.49	35.8±1.02	37.6±0.33	UNDET
EAV Detection	24.8±0.02	25.9±0.49	26.3±0.41	26.3±0.41	25.4±0.29	ND

Table 5: Equine Arteritis Virus (EAV) is used as an internal extraction and amplification control. A 1:50 dilution of EAV was spiked into the BAL samples along with 0.1pg, 1pg, 10pg, 100pg, 1ng, and 10ng of hMPV RNA. hMPV was also extracted and amplified without EAV. Numbers represent the Ct value +/- SD of 2 experiments. UNDET is undetermined and ND is not done.

4.3. hMPV Real Time NASBA Optimization:

bioMérieux has developed a real time NASBA assay for hMPV detection, and our lab has had the opportunity to evaluate this test on lung transplant recipients. Optimization of the assay was performed by bioMérieux and all information regarding the assay was proprietary, so the target was unknown. The primers, beacon, and KCl concentrations were defined with no room for adjustment, so the assay was performed according to bioMerieux's instructions. The negative cut-off value was defined in our laboratory by testing 140 negative patient specimens, per PCR. The mean Δvalue from each reaction was multiplied by the ratio of max/min [Mean Δvalue x (maxΔvalue / minΔvalue)] to get a cut off Δvalue of 0.041.

4.4. Construction of N Gene Plasmid:

A plasmid containing the entire hMPV N gene was constructed and used to produce RNA run-off transcripts. This RNA was then used as a positive control for the quantitation of hMPV by

real time RT-PCR. Known copy number of hMPV RNA is amplified as a standard curve during every experiment in order to quantitate positive specimens. This plasmid was constructed by ligating a 1250 bp PCR fragment containing the Can97-83 N gene into a pGEM®-T Easy Vector (Figure 6). The N gene was amplified by conventional two step RT-PCR using primers upstream and downstream of the gene (Figure 6B).

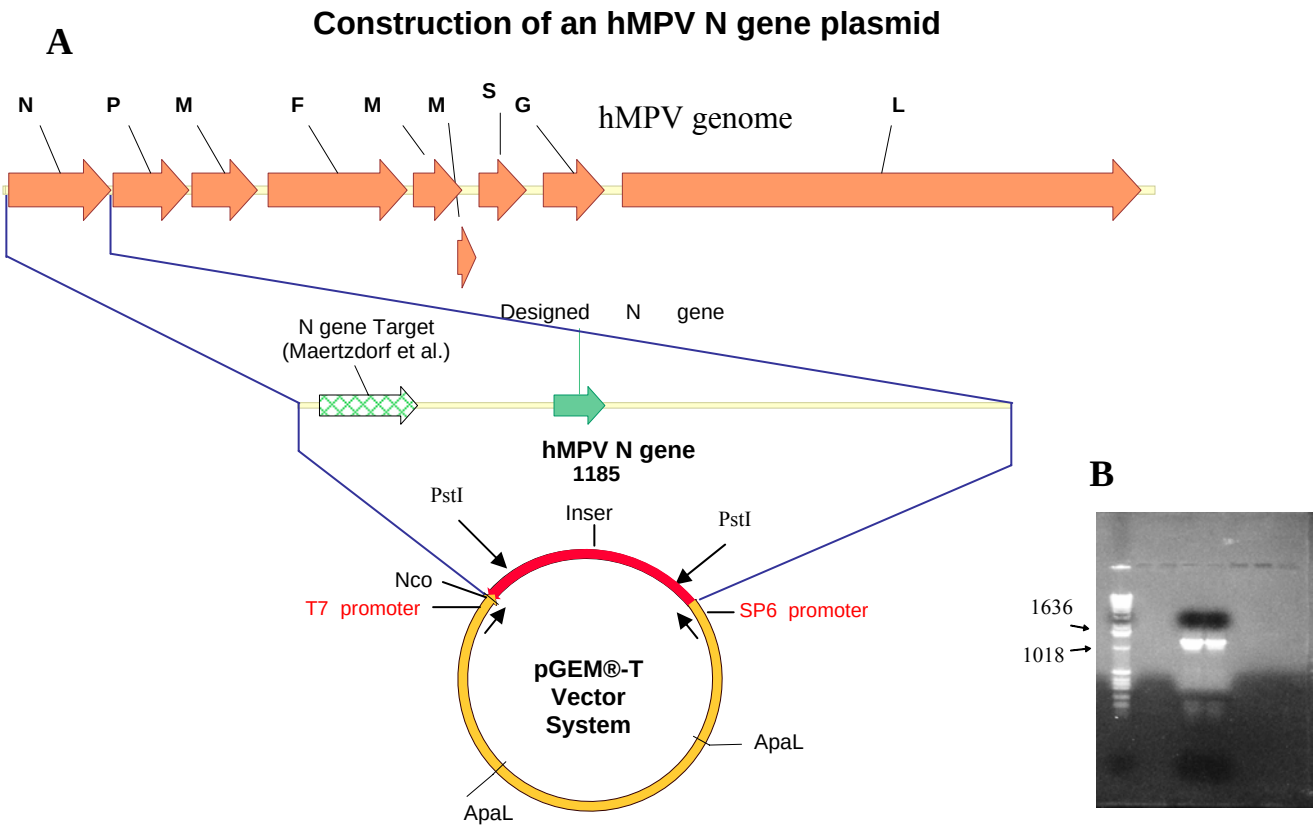


Figure 6: Schematic of construction of an hMPV N gene plasmid under the control of either SP6 or T7 depending on the orientation, A. The cloned N gene amplicon is 1250bp shown on a 1% agarose gel in duplicate, B. Note the Pst I sites within the insert and the NcoI site on the vector adjacent to the insert.

The PCR fragment was gel purified and ligated into a pGEM®-T Easy Vector overnight which was then transformed into JM109 cells. Plasmid DNA was extracted from twelve selected colonies thought to be expressing the plasmid. Of the twelve colonies picked, three of them actually contained the insert as evidenced by the presence of a 950bp fragment following a Pst I

digest (Figure 7A). The insert contains two Pst I restriction sites 950bp from each other (Figure 6A). The orientation of the insert is important because it will determine which RNA polymerase will be used for the transcription reaction. To determine the orientation of the insert, a double digest with Sac I and Hind III will yield either an 850bp fragment or a 620bp fragment (Figure 7C). Two of the three colonies (colonies 9 and 10) had the insert in the 3`-> 5 directions under control of the SP6 promoter. Colony 12 had the insert in the 5`->3` direction under control of the T7 promoter. (Figure 7B)

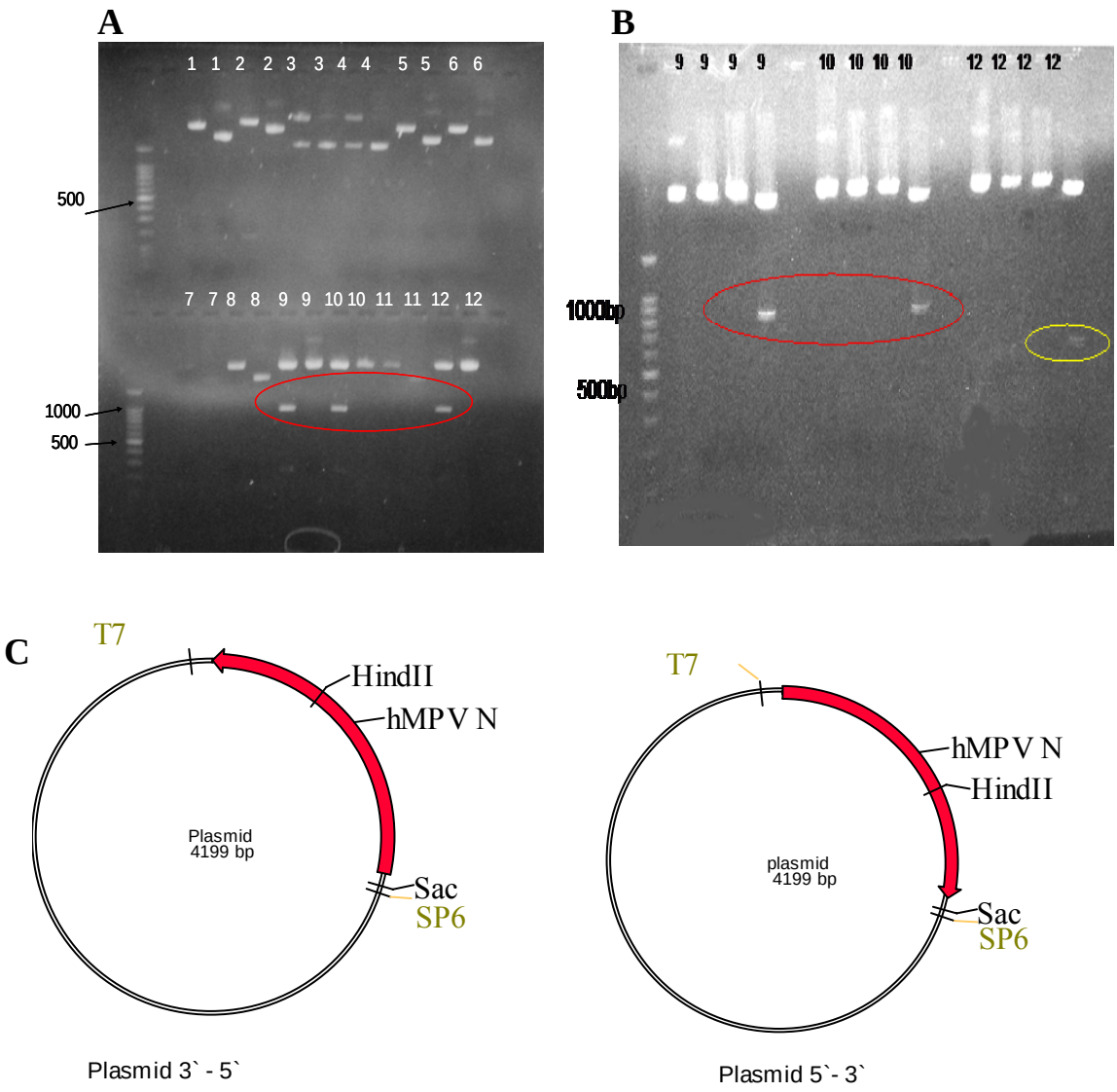


Figure 7: N gene plasmid DNA. The presence of a 950bp fragment indicates efficient ligation and transformation of the insert, three of the twelve picked colonies had the insert present (circled in red). The plasmid DNA was ran on a 1% agarose gel in TE buffer in the cut and uncut conformation respectively, A. Orientation of the inserts were determined by performing a double digest with Hind III and Sac I, two of the three were in 3`->5` direction under the control of the SP6 promoter and the 12th colony is in the 5`->3` orientation under control of the T7 promoter, B,C.

The original 9th colony in the 3`->5` orientation (Figure 8A) and the original 12th colony in the 5`->3` orientation (Figure 8B) were transformed and cloned again in order to make a stock of each plasmid for each orientation.

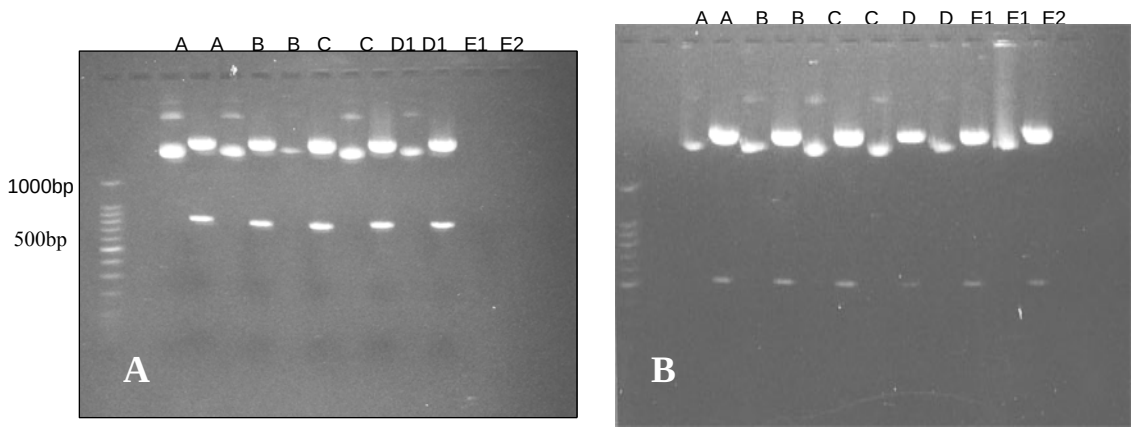


Figure 8: Plasmid DNA from colonies, A-E2, are from the 9th (A) and 12th (B) original colonies producing an 850bp band and a 620bp band respectively when double digested with SacI and Hind3. The bands on the left are uncut plasmid DNA and the bands on the right have been double digested. 12th colony: all 5 colonies have 620bp band. Left bands are uncut, Right bands are cut

In vitro transcription of the N gene was accomplished using the Ribomax™ Large Scale RNA Production System. The plasmid was linearized cutting the vector portion of the plasmid just 3` of the N gene insert using an Nco I site. Under control of the SP6 promoter RNA transcripts were made solely of the hMPV N gene (Figure 6A). The run-off transcripts were quantified by measuring the A260 using a spectrophotometer yielding a final concentration of 4.563×10^{12} copies/ul.

4.5. Limit of Detection for N Gene Real Time RT-PCR:

The hMPV real time RT-PCR targeting the N gene was developed to detect all four genetic lineages of the virus. To determine the capability of the test to detect various strains of the virus, a limit of detection assay was performed using the two main lineages, Can97-83 and Can98-75, for strains A2 and B2 respectively (Figure 9). Half log intervals were amplified from 100,000-

10 copies for both viruses and showed comparable limits of detection for the two strains detecting 50 and 100 copies respectively (Table 6).

N gene hMPV Real time RT-PCR Limits of Detection

hMPV Can97-83 (Strain A) hMPV Can98-75 (strain B)

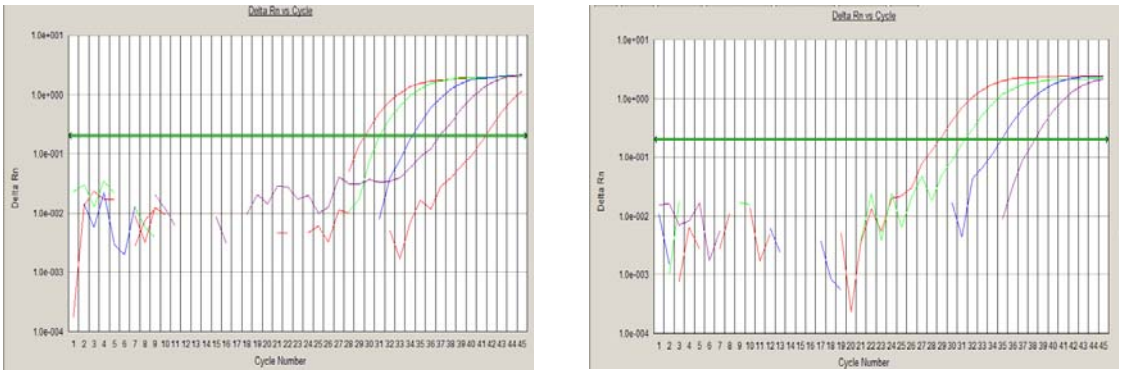


Figure 9: Amplification plots of serially diluted hMPV strains A (left panel) and B (right panel) detected by real time RT-PCR using the N target. 100,000 copies (red left), 10,000 copies (green), 1,000 copies (blue), 100 copies (purple), and 10 copies (red right).

4.6. Limit of Detection for Real Time NASBA:

hMPV strains A and B were amplified using real time NASBA with discordant limits of detection between the two main lineages. The NASBA assay could detect as low as 50 copies of strain A2 yet only 1000 copies of strain B2 (Table 6). Figures 10 and 11 show the actual amplification plots for serial dilutions of hMPV strains A and B. The fluorescence of each amplification from the serial dilutions was plotted against time in figure 12.

NASBA detection of hMPV strain A

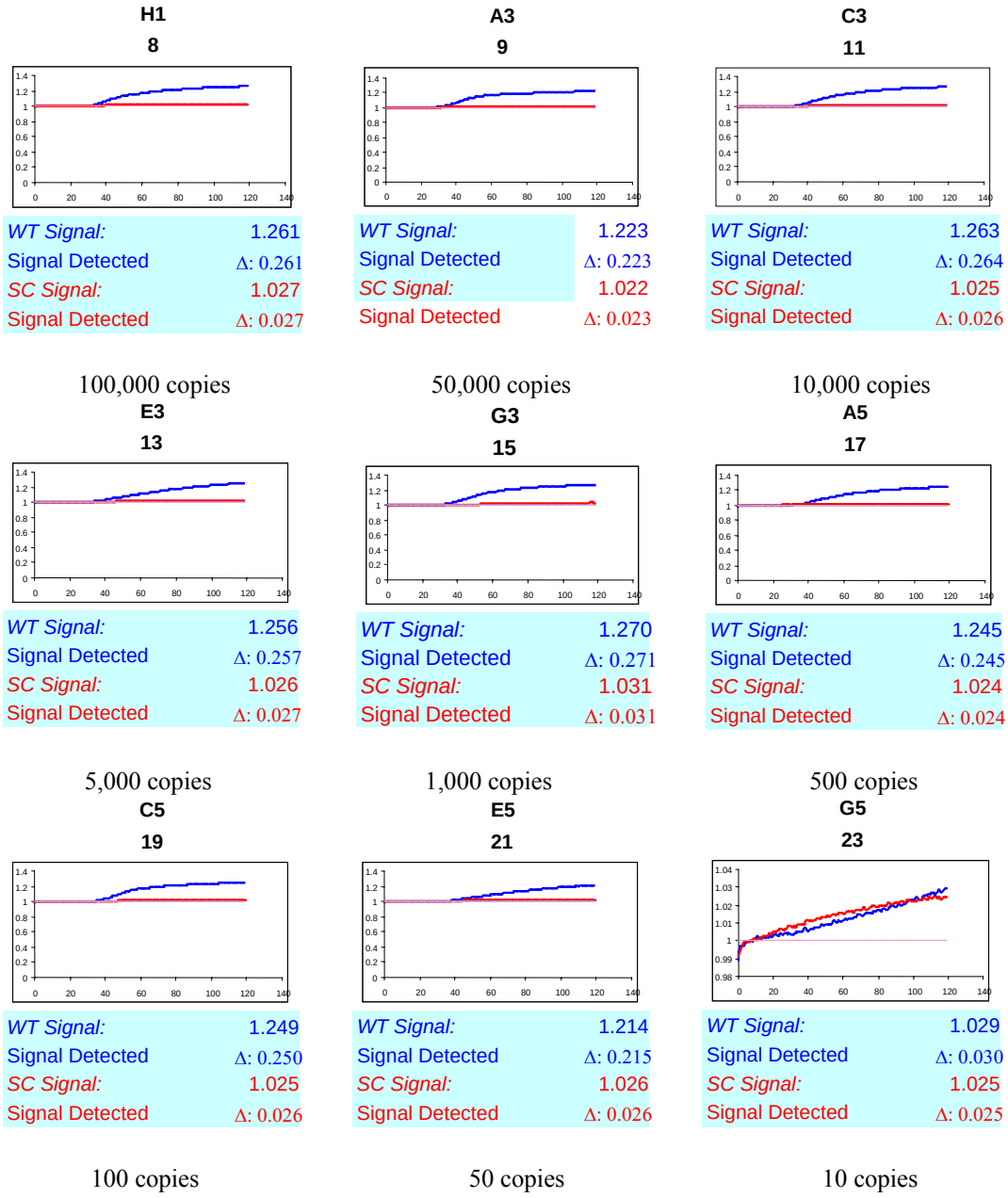
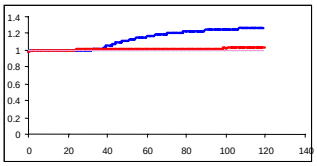


Figure 10: Real time NASBA limit of detection assay for hMPV strain A. This is not a quantitative test however the negative cut-off is Δvalue *0.041.*

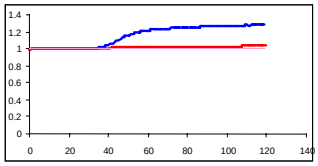
NASBA detection of hMPV strain B

A7
25



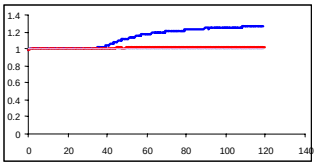
WT Signal:	1.263
Signal Detected	Δ: 0.263
SC Signal:	1.028
Signal Detected	Δ: 0.029

C7
27



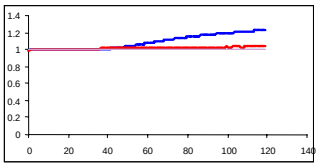
WT Signal:	1.283
Signal Detected	Δ: 0.283
SC Signal:	1.033
Signal Detected	Δ: 0.033

E7
29



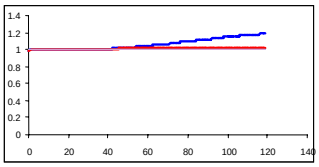
WT Signal:	1.267
Signal Detected	Δ: 0.267
SC Signal:	1.027
Signal Detected	Δ: 0.028

100,000 copies
A9
33



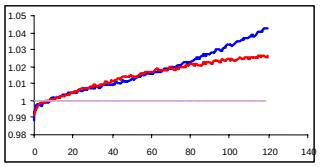
WT Signal:	1.226
Signal Detected	Δ: 0.227
SC Signal:	1.034
Signal Detected	Δ: 0.034

50,000 copies
C9
35



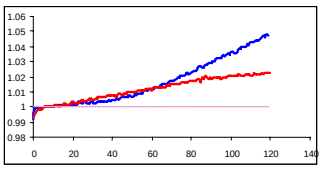
WT Signal:	1.186
Signal Detected	Δ: 0.187
SC Signal:	1.027
Signal Detected	Δ: 0.027

10,000 copies
E9
37



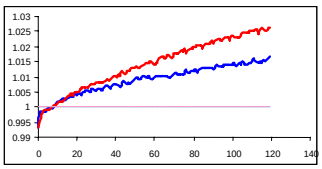
WT Signal:	1.042
Signal Detected	Δ: 0.043
SC Signal:	1.026
Signal Detected	Δ: 0.027

5,000 copies
A11
41



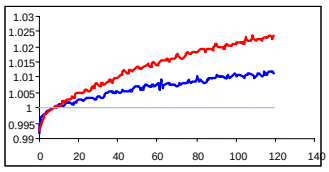
WT Signal:	1.048
Signal Detected	Δ: 0.048
SC Signal:	1.022
Signal Detected	Δ: 0.023

1,000 copies
C11
43



WT Signal:	1.016
Signal Detected	Δ: 0.017
SC Signal:	1.026
Signal Detected	Δ: 0.027

500 copies
E11
45



WT Signal:	1.011
Signal Detected	Δ: 0.012
SC Signal:	1.024
Signal Detected	Δ: 0.024

100 copies 50 copies 10 copies

Figure 11: Real time NASBA limit of detection assay for hMPV strain B. This is not a quantitative test however the negative cut-off is Δvalue *0.041.*

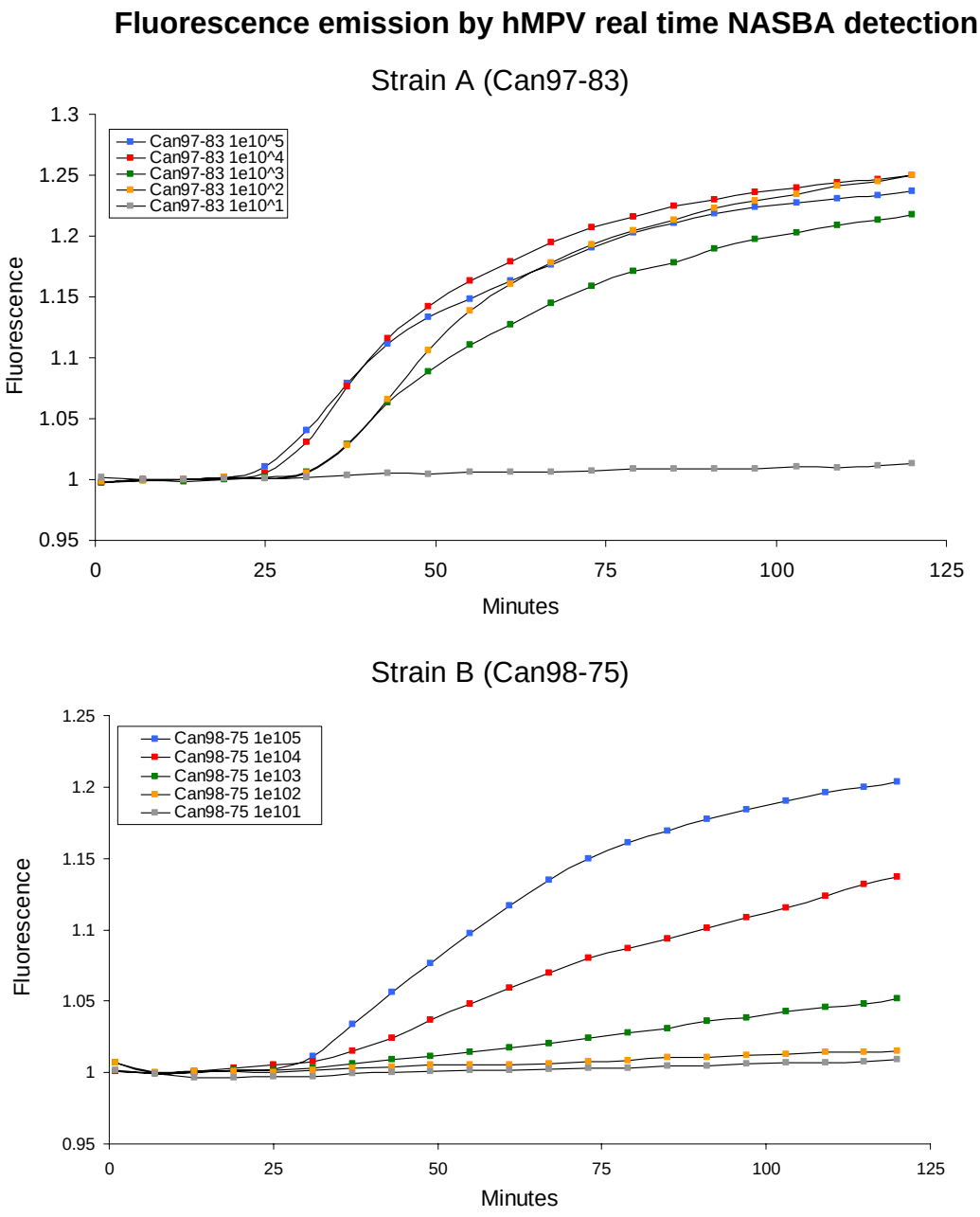


Figure 12: Fluorescence plot of samples from a serial dilution of hMPV detected by real time NASBA. Strain A (top panel) is more sensitive than strain B (bottom panel).

Table 6: Limits of Detrection for real time RT-PCR and real time NASBA

Assay	Strain	100000 copies	50000 copies	10000 copies	5000 copies	1000 copies	500 copies	100 copies	50 copies	10 copies
RT-PCR	Can 97-83	+	+	+	+	+	+	+	+	-
	Can 98-75	+	+	+	+	+	+	+	-	-
NASBA	Can 97-83	+	+	+	+	+	+	+	+	-
	Can 98-75	+	+	+	+	+	-	-	-	-

Table 6: Comparison of the real time RT-PCR and real time NASBA limits of detection for the amplification and detection of hMPV.

4.7. Specificity of the hMPV N Gene Real Time RT-PCR Assay:

The hMPV N gene real time RT-PCR assay was developed to detect all four genetic lineages of the virus. To ensure that false positives do not occur, a panel of respiratory virus RNA was assayed for amplification. Using the N target, RNA from influenza A and B virus, respiratory syncytial virus, parainfluenza virus 1-3, adenovirus, and SARS-CoV failed to amplify, indicating that this assay is specific to hMPV alone.

4.8. Detection of hMPV in Adult Lung Transplant Recipients:

A total of 234 BAL specimens from 114 lung transplant recipients were tested for hMPV infection by both real time RT-PCR and real time NASBA. Samples were collected from 5-13-04 to 4-11-05 spanning an 11 month period. The patient's ages ranged from 22-75 years old with an average age of 51.7. The cohort was comprised of 41.2% males and 58.8% females. 3 specimens were positive for hMPV by RT-PCR (1.28%) including one specimen that was not

detected by NASBA; however, NASBA detected 4 (1.71%) hMPV positive specimens, including 2 that were not detected by RT-PCR. There was a total of 5 samples (2.14%) from different patients positive for hMPV all occurring during the late winter season between 2-17-05 and 4-1-05 (Table 7). All samples included in the study extracted and amplified without inhibition as evidenced by positive EAV Ct values (data not shown).

An immunofluorescence assay was used to determine the prevalence of other respiratory viruses in the lung transplant recipients; flu A (0%), flu B (0%), RSV (0%), PIV1-3 (0%), and Adenovirus (0.0043%). In this patient population, hMPV was the most prevalent respiratory viral infection (2.14%). Two patients, 1124 and 1119, were positive for CMV antigenemia from the BAL specimens, which were also positive for hMPV. Patient 1130 was the only positive patient during a routine follow-up, the rest of the positive patients underwent bronchoscopies due to respiratory symptoms. Patient 1119 was rejecting the graft at the time of hMPV infection. Patient 1396 was positive for hMPV in addition to *Candida albicans, Streptococcus viridans, Neisseria,* and *Propionibacterium Diphtheroids* infections. Patient 1130 was positive for hMPV on 2/17/05 and then negative on 4/4/05 indicating that the infection cleared and is not persistent. During this time, the patient was positive for hMPV in addition to *Candida albicans* and *Streptococcus viridans*.

This study was on specimens taken throughout the entire year. hMPV infection is known to be most prevalent during the late winter months (February-April). In this cohort, the prevalence of hMPV infection is 9.6% during this time period establishing it as a prevalent pathogen in this patient population during the late winter months.

Table 7: hMPV positive lung transplant recipients

Pt ID	Date	PCR	Copies/ml	NASBA	IC
1119	4/1/2005	Pos	11,865	Neg	Pos
1124	3/15/2005	Pos	18,895	Pos	Pos
1130	2/17/2005	Pos	12,100	Pos	Pos
1396	2/22/2005	Neg	-	Pos	Pos
1422	3/23/2005	Neg	-	Pos	Pos

Table 7: List of hMPV positive patients from the lung transplant cohort. Quantitation of specimens were only done on PCR positive. Internal Control (IC) were all positive.

4.9. Detection of hMPV in Symptomatic Children:

328 nasopharyngeal (NP) swabs taken from children during the 2004-2005 winter season were tested for *Bordetella pertussis* at Childrens Hospital of Pittsburgh (CHP). Using these specimens, a retrospective study was designed to look for hMPV infection by real time RT-PCR. The specimens were collected by doctors, nurses, and other health care providers at CHP in the emergency department, nursing units, and fast track unit as well as from other institutions such as Latrobe Hospital, West Penn, Mercy Hospital, and Molecular Diagnostics (UPMC). Samples were collected from 09-09-2004 until 02-01-2005 during the early and middle winter season. Samples for late winter months could not be obtained due to requirements of CHP not releasing samples until 3 months after the collection date. The patients ranged in age from 14 days to 59 years old averaging 6.65 years. The study included 53.5% male and 46.5% female patients.

There were only 6 (1.83%) reproducible positives by real time RT-PCR and 2 of these were also positive for *Bordetella pertussis* indicating a coinfection (Table 8). 7 other patients tested positive for hMPV, however, showed very low levels of RNA near the limit of sensitivity and were not reproducible. Due to the lack of hMPV NASBA reagents provided by bioMérieux, we were unable to assay all of the patient specimens by this detection method. However, the 6

reproducibly positive RT-PCR samples were tested for hMPV by NASBA and only 2 of these patients were positive (Table 8).

45 (13.7%) of the specimens were positive for *Bordetella pertussis* making it the most prevalent pathogen present in this cohort. 30 specimens from this study were tested for other respiratory viruses indicating the following prevalence's; Flu A (3.3%), Flu B (0%), RSV (3.3%), PIV1-3 (0%), Rhinovirus (3.3%) and Adenovirus (3.3%). These other respiratory specimens were detected by culture methods so the detection may not be as sensitive as the hMPV PCR. Real time RT-PCR detected hMPV in 6.67% of these same 30 specimens.

Table 8: hMPV positive patients suspected of *Bordetella pertussis*

Pt ID	Date	Age	*B. pertussis*	hMPV PCR	Copies/ml	hMPV NASBA	IC
78	10/18/2004	40Y	Neg	Pos	112,351	Neg	Pos
179	11/28/2004	21M	Neg	Pos	43,148,150	Neg	Pos
217	N/A	7Y	Pos	Pos	1,948,864	Neg	Pos
299	1/12/2005	10M	Pos	Pos	3,819,584	Pos	Pos
309	1/16/2005	26M	Neg	Pos	8,227,103	Neg	Pos
311	1/18/2005	7M	Neg	Pos	149,000,000	Pos	Pos

Table 8: List of hMPV positive patients from CHP study. Coinfection occurred in 2 of these patients. Internal Control (IC) were all positive.

5. Discussion

This newly described paramyxovirus has been detected throughout the world and has come to be known as a major cause of community acquired respiratory infections. Since its discovery in 2001, hMPV has been found primarily in children, though elderly and immunosuppressed individuals have shown to be susceptible to infection. Recently, it has been established that there are four different genetic lineages of hMPV and all four circulate at the same time with no evidence geographic clustering or antigenic drift. Because of this, a diagnostic test capable of detecting all lineages and strains of the virus is essential for proper diagnosis such that infections are not missed due to assay bias. In this study, a real time RT-PCR assay was designed and developed to detect all lineages of the virus and it was compared to an hMPV real time NASBA assay designed by bioMérieux which is currently in developmental stages of production. Our laboratory has been working in collaboration with them to evaluate their assay and test patient specimens since they do not have easy access to patient samples. They provided us with amplification kits and hMPV sequence specific primers and a molecular beacon but have just optimized the internal control (IC) for this assay, so it was not included in the study. This IC needs to be used prior to extraction and amplification and then validated for real time NASBA. Both tests were used to detect hMPV infection in lung transplant recipients as well as symptomatic children suspected of *Bordetella pertussis*.

The RT-PCR was designed to target conserved sequences among all four strains of the virus. Degenerate bases were used when able to account for variability between the strains in order to detect as many infections as possible. The degenerate bases noted by an R indicate either an A or G in the position such that 50% of the synthesized primers will contain an A and the other 50% will contain a G. The degenerate bases noted by a Y indicate either a C or T in the position

such that 50% of the synthesized primers will contain a C and the other 50% will contain a T. Five real time RT-PCR assays were developed and in combination with a published set (31) were all compared for strain sensitivity and overall ability to detect virus.

The Maertzdorf primer/probe set (31) appeared to detect hMPV better than the designed MGB sets based on high quantity strain A2 RNA (Table 3). When comparing sensitivities of the various strains, this published set failed to amplify lower levels of hMPV RNA, though they claim to be able to detect as low as 10 copies of RNA (Table 4). Primers and probe were ordered a second time to make sure they were not poorly synthesized oligonucleotides and the same problems occurred. This discrepant report is probably due to the use of different molecular platforms. They used a universal mastermix from a different company than used in our lab and the PCR conditions varied as well as the primer/probe concentrations. Our amplification parameters and one step mastermix must remain consistent such that we can amplify many targets at one time without having to change conditions.

The N gene target utilizing an MGB molecular probe has the best overall sensitivity compared to the other RT-PCR assays in addition to the NASBA assay developed by bioMérieux (Figure 4 and Table 6). The N gene RT-PCR as well as the NASBA assays can detect as low as 10 copies of strain A2 RNA however, this limit of detection is not always reached so it is reported that the assay can only detect 50 copies of hMPV RNA (Figure 9). The ability of the RT-PCR assay to detect hMPV viral RNA in actual specimens was tested by spiking whole virus into BAL specimens prior to extraction. The virus was detected more efficiently when extracted in actual BAL samples compared to lysis buffer (control matrix) (Figure 5). Both of these sample types were compared to the sole amplification without extraction of hMPV RNA. There is an expected loss of viral RNA during the extraction procedure as reflected by a slight increase in Ct value for

these samples. This indicates that hMPV can be detected in patient specimens using the automated extraction method we are currently using in our laboratory.

The use of an adequate internal control is imperative for molecular based assays. A non human virus can be used as a quantitative internal control because a known amount is spiked into each specimen prior to extraction. In addition, using a virus mimics the extraction and amplification of the target virus (hMPV). Equine arteritis virus (EAV) is used in this system to determine if there is any form of inhibition throughout the extraction and amplification process. The average Ct value+2SD of 100 EAV spiked samples set the cut-off value for inhibition at a Ct value of 30 or higher. This internal control served as a means to determine when a negative sample should be repeated.

For a quantitative real time RT-PCR assay, every experiment contains a serial dilution of positive material in order to accurately quantitate positive specimens. For this reason, sufficient amounts of control material will be needed for future testing since this assay was developed with the intention to go on-line at the University of Pittsburgh Medical Center (UPMC) as a diagnostic test in the clinical virology laboratory. Construction of a plasmid containing the target sequence was completed which can be used for production of RNA runoff transcripts producing high copy number of the gene of interest. A plasmid containing the entire N gene was constructed due to the difficulty of culturing hMPV and the lack of commercially available hMPV RNA.

Though hMPV has been detected in immunosuppressed individuals, there has yet to be a study determining the prevalence of hMPV in lung transplant recipients; this is the first report of such a study. The University of Pittsburgh Medical Center is a global leader for solid organ transplants. This institution performs more than fifty lung transplants per year and follows over two hundred

fifty lung transplant recipients for routine post transplant examination. BAL samples are collected from lung transplant recipients at 3, 6, 9, and 12 months post transplant, as well as every 6 months after that for a maximum of 3 years. In addition to routine collection, specimens are also collected from symptomatic transplant recipients with possible respiratory infections. BAL specimens from lung transplant recipients were collected at routine visits in addition to visits due to symptomatic events.

In this one year study, 2.14% of all patients were positive for the virus. Though this is a low percentage, these specimens were collected all year long, though hMPV is a late winter virus. In addition, many of these patients have come to see the physician because of routine post transplant follow-up and have no signs or symptoms of respiratory viral infection. When monitoring the late winter months, 9.6% of the patients were positive for hMPV establishing it as a prevalent pathogen in this patient population during the late winter months. Every specimen was tested for other respiratory viruses by an immunofluorescense assay; however all were negative except one adenoviral infection.

The lack of other respiratory viruses indicates that hMPV could be a major pathogen in this population.

Knowing that this virus primarily infects children, this assay was used to detect hMPV in NP swabs from children suspected of *Bordetella pertussis*. Patients infected with pertussis present with a broad range of symptoms indicating there could be a secondary infection due to the pertussis infection. It is possible that pertussis infection along with a respiratory viral infection may result in a series respiratory illness. In this study only 1.83% of the specimens were positive for hMPV however 13.7% were positive for *Bordetella pertussis* (Table 8). In addition 2 (33.3%) of the 6 positive patients were co-infected with *B. pertussis*. There were 7 patients that

were detected as positive for hMPV then when re-amplified, they failed to amplify. This is due to the limit of detection not being able to amplify RNA of low levels on a consistent basis. All of the positives that did not re-amplify had Ct values of 38-44. bioMérieux did not provide new reagents for this study so all 328 were not tested for hMPV by real time NASBA. However, there were enough reagents left over to test the RT-PCR positives by NASBA, and only 2 were positive (Table 8). There is future work to do with this project starting with sequencing all patient positives to determine the strain prevalence in Pittsburgh.

This pediatric study was cut short by not being able to access the samples from February to April (the peak hMPV months). I feel that if the study could have accessed these samples prior to three months after specimen collection, a higher incidence of hMPV infection would have been noted. A majority of the patient positives were in January as were the non-reproducible positives. Only 30 of these patient specimens were cultured for other respiratory viral infection. Of these, one specimen was positive for rhinovirus (3.3%), one was positive for adenovirus (3.3%), one was positive for influenza A (3.3%) virus, and one was positive for RSV (3.3%). Out of this group of patients, 2 were positive for hMPV PCR (6.6%). Though a small number of patients were included, hMPV was the most prevalent respiratory virus in the group. This study should proceed with testing the patient specimens for hMPV during the late winter months of February-April.

In conclusion a real time RT-PCR assay was developed to detect all four lineages of hMPV targeting a conserved region of the N gene. This assay was compared to an hMPV real time NASBA in the developmental stages of production by bioMérieux. The limits of detection for the RT-PCR for both of the main lineages are similar when comparing Can97-83 (Strain A2) and Can98-75 (Strain B2) control material. Alternatively, the real time NASBA detects hMPV viral

RNA from the A strain with high efficiency yet the limit of detection is not as sensitive from the strain B. When comparing the two assays in patient studies, each of the diagnostic tests detected viral infections that failed to be detected by the other molecular based method (Table 7). The NASBA could only detect 33% of the hMPV positive patients as indicated by RT-PCR in the CHP study. hMPV was detected at a low prevalence in lung transplant recipients (2.14%), however, during the late winter months the prevalence of hMPV goes up to 9.6% indicating that this virus is seasonal and may play an important role as a respiratory pathogen during this time period. hMPV was detected in children suspected of pertussis at an expected low level of prevalence (1.83%). However, this study only includes patient specimens from September to January. The peak season for hMPV is February to April. A majority of the positives from this study, including the non reproducible positives were in January. This prevalence would be expected to increase during these late winter months. hMPV is a seasonal respiratory virus which infects children and immunosuppressed individuals and can be detected by molecular based methods.

This research is of major public health significance due to the amount of respiratory infections that are going undiagnosed or being treated with unnecessary antibiotics. It is important for our physicians to not only know that hMPV is present in our community but also to be able to detect it. This study reports the first evidence of hMPV in the Pittsburgh area and demonstrates the importance of this virus as a critical player among respiratory pathogens in both immunosuppressed lung transplant recipients and children.

Future work on this project includes; completing the pediatric study through the winter season to get an appropriate prevalence of hMPV in this patient cohort, repeating previous positives to determine the reproducibility of the low level positives for this assay, sequencing the positive

patients to determine which genetic lineages are being missed by certain molecular assays and to determine if there are certain strains more prevalent than others within our community, and to attempt to culture the patient positives by cell culture to grow up a virus stock and confirm PCR based positives.

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