DEVELOPMENT OF A STANDARDIZED QUANTITATIVE REAL TIME PCR PANEL FOR RESPIRATORY VIRAL DIAGNOSIS

by

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ABSTRACT

Traditional viral diagnostics such as viral culture and various serological techniques tend to be slow, insensitive and labour-intensive. A large proportion of viral pathogens still go undetected using these techniques. This thesis concerns the development of a rapid and sensitive technique – standardized real-time quantitative PCR. Individual qPCR assays and synthetic plasmid controls were developed for 12 common respiratory viruses including influenza types A and B, parainfluenza (PIV)-1, -2 and -3, respiratory syncytial virus (RSV) A and B, metapneumovirus (MPV), human coronavirus (HCoV) 229E and OC43, human rhinovirus (HRV) and adenovirus. A reference gene assay using hypoxanthine phosphoribosyl transferase (HPRT) was also developed. A retrospective analysis on nasopharyngeal aspirates from patients previously diagnosed was conducted. The results demonstrated that the respiratory viral qPCR panel was sensitive, efficient, and had a large dynamic range of detection. Some cross-reactivity was noted for HRV with an enterovirus (coxsackievirus B3). HPRT proved to be a stable reference gene with the additional benefit that qPCR viral loads could be interpreted based on copy number per unit volume of specimen. One hundred culture negative specimens were examined and viral nucleic acid was amplified in 43 of them. There was a statistically significant relationship between viral load and whether or not the same specimen was positive by culture for influenza A, PIV-3, RSV A and B, HRV and adenovirus. Mean viral load was highest in patients with lower respiratory tract infections (LRTI) compared to those with fever or upper respiratory tract infections (URTI) and 95% confidence interval (CI) between these patients did not overlap. These results suggest that patients with more severe clinical disease had higher viral loads. This study highlights the developmental

phase of a technique that has the potential to increase the detection rate of viral pathogens involved in respiratory illnesses.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

ABBREVIATIONS

ACE2 ..... angiotensin converting enzyme 2

CAR ..... coxsackie adenovirus receptor

CD ..... cluster of differentiation

CFT ..... complement fixation test

CPE ..... cytopathic effect

CVB3 ..... coxsackie virus 3

DNA ..... deoxyribonucleic acid

DTT ..... dithiothreitol

ELISA ..... enzyme linked immunosorbent assay

FAM 6-carboxyfluorescein

F ..... fusion (protein)

FRET ..... Forster resonance energy transfer

G ..... glycosylated (protein)

GAPDH ..... glyceraldehyde-3-phosphate dehydrogenase

HA ..... hemagglutinin

HAI ..... hemagglutinin inhibition assay

hAPN ..... human aminopeptidase N

HCoV ..... human coronavirus

HE ..... hemagglutinin-esterase

HEK ..... human embryonic kidney

HLA I ..... human leukocyte antigen I

HN ..... hemagglutinin-neuraminidase

HRV ..... human rhinovirus

HPRT ..... hypoxanthine phophoribosyl transferase

ICAM-1 ..... intracellular adhesion molecule-1

LB media ..... Luria-Bertani media

IF ..... immunofluorescence

LDL-R ..... low density lipoprotein receptor

LRTI ..... lower respiratory tract infection

MDCK ..... Madin-Darby canine kidney

MHC-I ..... major histocompatibility complex-I

MK ..... monkey kidney

MPV ..... metapneumovirus

M ..... membrane (protein)

NA ..... neuraminidase

NCBI ..... National Center for Biotechnology Information

NS ..... non-structural (protein)

NPA ..... nasopharyngeal aspirate

NPS ..... nasopharyngeal swab

OPS ..... oropharyngeal swab

PCR ..... polymerase chain reaction

PMK ..... primary rhesus monkey kidney

PIV ..... parainfluenza virus

RNA ..... ribonucleic acid

ROX ..... carboxy-X-rhodamine

RSV ..... respiratory syncytial virus

RT-PCR ..... reverse transcriptase polymerase chain reaction

S ..... spike (protein)

SARS ..... severe acute respiratory syndrome

SH ..... small hydrophobic (protein)

SURE® cell ..... Stop Unwanted Rearrangement Events cell

TAMRA 6-carboxy-tetramethyl-rhodamine

URTI ..... upper respiratory tract infection

VP ..... viral protein

1. RESPIRATORY VIRUSES

1.1 Historical Perspectives

Viruses are obligate intracellular parasites that are dependent on the metabolic and genetic functions of living cells for survival. As they do not encode genes for ribosomal proteins or enzymes involved in energy metabolism, they must utilize the host cells' machinery to reproduce. The ancestry of viruses is ambiguous since they have only been discerned and categorized in the last century. The first clue of the existence of viruses was uncovered in 1879 by Adolf Mayer, a German scientist, who was credited as being the first person to transmit a plant pathogen from liquid extracts of a diseased plant to a healthy one (1). In 1892, Russian scientist Dmitri Ivanowski showed that tobacco plants, which were susceptible to what is now known as tobacco mosaic disease, became infected and died after exposure to a filtered substance which he thought caused the plant infection (2). In 1898, Dutch scientist Martinus Beijerinck refined Ivanowski's experiments by filtering out this infectious agent and heating it. Through his experiments, he was able to rule out chemical toxins because the agent reproduced and proliferated in plant cells (3). Bacteria were also excluded because the filter used was fine enough to prevent passage of bacteria as the disease causing agent. Ivanowski instead named the agent "contagious living fluid" which was later renamed "virus", the Latin word for poison. Shortly after, the first animal virus, known as foot and mouth disease (4), was identified in cattle by German bacteriologists Friedrich Loffler and Paul Frosch, two trainees of Robert Koch, a German physician who is considered one of the forefathers of bacteriology. The discovery of the first human virus was that of yellow fever in 1900 by American bacteriologist Walter Reed and colleagues (5).

Scientists were only able to describe a virus by the symptoms or disease it caused; structurally, viruses were an unknown entity. In fact, dissent amongst scientists remained for well over twenty years as to whether viruses were particles, liquids or something else. Various findings in the following years helped to finally elucidate the nature of viruses. In 1917, Canadian scientist Felix d'Herelle discovered viruses that could infect bacteria, which he termed bacteriophages (6). He demonstrated that these bacteriophages could make holes ("plaques") in a bacterial culture. Since each plaque was cultivated from a single bacteriophage, this provided a method of counting the number of infectious viruses and was the forerunner of the plaque assay. Further analyses of bacteriophages in the 1940s and 1950s established certain properties (i.e., size, shape, and morphology) due to the ease of growing these bacterial viruses in the culture (7, 8). In 1935, American scientist Wendell Stanley crystallized the tobacco mosaic virus and demonstrated the virus had a definite, consistent shape (9). Only in the late 1930s, after the invention of the electron microscope, were viruses able to be visualized.

1.2 Discovery of Respiratory Viruses

Concerning viruses that have a propensity to infect the respiratory tract, influenza was first characterized in swine in 1931 by American bacteriologist Richard Shope (10). What eventually came to be known as influenza type A, was subsequently isolated (a purified sample containing no other particulates) from a human in 1933 by English physicians Wilson Smith, Christopher Andrewes and Patrick Laidlaw (11). Both viruses were antigenically similar and serologically related to the influenza virus of the 1918 pandemic. Type B (12) and C (13) influenza viruses were isolated by Thomas Francis Jr. and Thomas Magill in 1940 and by Richard Taylor in 1949, respectively.

The 1950s and 1960s saw a rapid increase in the number of human respiratory viruses discovered. The four human parainfluenza viruses (PIV) were first identified in the late 1950s by Robert Chanock (14-19). He also discovered respiratory syncytial virus (RSV) in 1956 in infants with lower respiratory tract infections (LRTI) (20, 21). Adenovirus was accidentally isolated in 1953 by Wallace Rowe and colleagues who were trying to establish adenoidal cell lines from infected tissue of children who had tonsillectomies (22, 23). Human coronaviruses (HCoV) were discovered in 1965 by David Tyrrell and ML Bynoe in cultures of human ciliated embryonic trachea (24). In 1966, Dorothy Hamre and John Procknow characterized the 229E strain of HCoV which was grown successfully in WI-38 cells (25). In 1967, Kenneth McIntosh isolated six morphologically similar viruses and grew them in organ cultures; one of these was HCoV-OC43 (26). Human rhinovirus (HRV) was discovered in the 1950s by growing a strain of the virus in tissue culture and described separately by the Common Cold Unit in Great Britain and laboratories in the United States (27-29). It was not until 2001 that human metapneumovirus (MPV) was first isolated in cultures from hospitalized children in the Netherlands by Albert Osterhaus and colleagues (30).

There is much that is still unknown and left to be discovered as the underlying cause of a significant proportion of respiratory infections are still unaccounted for. A study by Stockton and colleagues determined that in patients who presented with influenza-like illness, approximately 50% of the specimens had no identifiable virus (31). Another recent study found that no pathogen could be detected in 30-40% of hospitalized patients exhibiting a respiratory tract infection (32). Therefore, it is quite possible that there are many more, as yet, undiscovered viruses circulating that cause serious respiratory disease. Current molecular techniques such as

microarrays, large scale sequencing, and bioinformatics have facilitated in the discovery of novel viruses, such as avian influenza virus in 1997 (33), HCoV-HKU1 (34) and -NL63 (35) in 2004, and bocavirus (36) as of early 2006. HCoV-NL63 has been reported to cause severe lower respiratory disease similar to croup in young children and in immunocompromised patients (37, 38). It is unclear at this point, how much of a worldwide impact these recently discovered respiratory viruses may have as their clinical manifestations and epidemiologies have yet to be elucidated. They may be common causes of respiratory infections as early epidemiological studies into MPV (39) have shown, or alternatively may be a rare infection like the severe acute respiratory syndrome (SARS) outbreak in 2003 – only time will tell.

1.3 Classification

Viral classification is generally based on five main criteria. These include: (1) the nature of nucleic acid genome (RNA or DNA); (2) number of strands of nucleic acid and their physical construction (single- or double-stranded, segmented or not); (3) polarity of the genome (negative (-) or positive (+) polarity); (4) presence or absence of a lipid envelope; and (5) shape (icosahedral, helical or complex). Respiratory viral infections are generally caused by single-stranded negative polarity RNA, single-stranded positive polarity RNA, and double stranded DNA pathogens.

1.3.1 Single Stranded Negative Polarity RNA Viruses

Orthomyxoviridae (influenza virus) and paramyxoviridae (PIV, RSV, and MPV) are the two family of viruses included in this group. They are all enveloped, helical viruses. Their names are derived from the word myxo meaning "an affinity for mucus", while ortho and para stand for

"true" and "closely resembling", respectively. The negative polarity of the RNA genome prevents the virus from being immediately infectious, in contrast to the positive strand RNA viruses. Positive strand viruses are of the same polarity as mRNA and are directly able to serve as templates for protein translation of viral proteins involved in genomic replication. Negative strand viruses go through the extra step of transcribing complementary full-length positive polarity RNA first.

1.3.1.1 Influenza Virus

Influenza virus is a medium-sized (80-120 nm diameter), enveloped, helical virus that is segmented into eight sections (seven for type C) that code for ten proteins (40). Segmented viruses have multiple RNA molecules enclosed in a single particle. The RNA is protected by the nucleocapsid which contains type-specific antigen that differentiates between strains, A, B, and C. The two genes that recombine to produce new strains of influenza are hemagglutinin (HA) and neuraminidase (NA), both found on the surface of the virion. The HA glycoprotein attaches to host sialic acid residues and allows the virus to bind and enter the cell, while NA cleaves those sialic acid residues and assists in releasing viral progeny from infected cells. These two antigenically recognized glycoproteins are used for determining the different serotypes of influenza A virus. Fifteen subtypes of hemagglutinin and nine subtypes of neuraminidase have been described, of which three subtypes of HA (1-3) and two subtypes of NA (1, 2) are endemic human influenza viruses. HA 5, 7, 9 and NA 7 have also been documented to infect humans (41). The prototype virus is named according to the type of virus, where the virus was isolated, specimen number and year (eg. A/HK/1/68 = type A, isolated in Hong Kong from specimen number 1 in 1968).

The genetic changes in nucleic acids that occur in a segmented virus like influenza is the root cause of re-infection and these antigenic changes determine the extent and severity of epidemics. These changes occur primarily in type A influenza and to a lesser extent, type B. By contrast, type C influenza is relatively stable and causes little to no illness in humans. A recent study by Matsuzaki and colleagues found only 187/84,946 (0.22%) influenza C infections over 14 years time (42). Two categories of genetic variation are involved. Antigenic drift arises when an accumulation of point mutations in the viral genome take place to generate HA and NA glycoproteins with minute differences that gives the virus new antigenic markers which evade the host's immune system. This leads to re-infection of the same virus but of a different strain and is sufficiently distinctive enough to cause an epidemic. The severity of the epidemic is dependent on the extent of antigenic variation.

The more severe type of variation, antigenic shift, occurs less frequently (43) and involves the mixing or reassortment of RNA segments of two different influenza viruses or human segments with those of avian or porcine fragments, creating a new serotype. Birds, humans and pigs possess receptors that recognize influenza. Migratory water fowl, in particular wild ducks, are the primary reservoirs of antigenically stable influenza viruses. In birds, influenza virus multiplies in the epithelial cells of the gastrointestinal tract and rarely causes illness. Virus gets excreted through feces which contaminate the water supply and other birds, such as chickens, may become infected through contact with contaminated water. Pigs are permissive, intermediate hosts to influenza; possessing both avian and human flu receptors and may become infected through contact with infected birds, humans or contaminated water. If avian and human influenza viruses infect a pig simultaneously, each independently replicating its nucleic acids and

proteins, segments from each may recombine into a chimeric virus with novel antigenic markers that are wholly unrecognized by the host's immune system. Antigenic shifts are therefore the cause of global pandemics since the majority of the population lack any immunity to fight these new strains, and are left vulnerable.

Four pandemics involving influenza A have been documented in the twentieth century based on serological studies. The 1918 Spanish flu pandemic was caused by H1N1 and was replaced in 1957 by H2N2, the Asian flu pandemic (44). In 1968, H3N2 emerged to cause the Hong Kong flu pandemic (45). Most recently, H1N1 re-emerged in 1977 as the Russian flu and circulates concurrently with H3N2 (46). The current concern is the H5N1 strain ("avian influenza") which is being closely monitored by the World Health Organization and the Centers for Disease Control and Prevention. It was first detected in Hong Kong in 1997 where 6 out of 18 patients died (33). Presently, the avian strain does not transmit easily from fowl-to-human or human-to-human. Recent studies elucidated why the virus is so lethal yet difficult to transmit (47, 48). The cellular receptor for influenza is a sialic acid linked to galactose by either an alpha-2, 3 or alpha-2, 6 linkage (49). It was recently determined that avian strains bind preferentially to an alpha-2, 3 conformation while human strains bind to an alpha-2, 6 linkage (48). These studies found that humans have both of these types of receptors; alpha-2, 6 receptors dominating the upper respiratory tract and alpha-2, 3 receptors found only in the lower respiratory tract in alveoli. This may explain the damage that occurs in the lower lungs in patients who have died (50). The current concern with most leading experts is that, over time, this avian strain will mutate to gain the ability to spread easily between humans. Since the population has no immunity to H5, it is

very likely that this strain will be extremely lethal if it becomes capable of person-to-person transmission (51).

1.3.1.2 Paramyxoviruses

Paramyxoviruses are enveloped and have a non-segmented and helical genome containing six basic genes and a subset of accessory genes that are unique to each genus (52). Paramyxoviruses transport their own RNA polymerase into the host cell, as these viruses cannot use the cellular machinery to replicate.

1.3.1.2.1 Parainfluenza Virus (PIV)

PIV is a large (120-300 nm diameter), non-segmented, enveloped, helical virus (52). It is differentiated antigenically into four subtypes and PIV-4 can be further subdivided into A and B strains. There are two antigenic proteins: hemagglutinin-neuraminidase (HN), a viral attachment protein which also allows the virus to adsorb and agglutinate erythrocytes, and fusion (F) which fuses the virion to the host cell.

1.3.1.2.2 Respiratory Syncytial Virus (RSV)

RSV is a large (120-300 nm diameter), non-segmented, enveloped, helical virus that has ten genes with code for eleven polypeptides (53). The envelope contains two antigenically recognized glycoproteins, fusion (F) and glycosylated (G), that stimulate the production of neutralizing antibodies. The F protein assists in viral entry and facilitates viral fusion to form syncytia while the G protein is believed to be responsible for the initial attachment of viruses to the host cell. Unlike other members in the family, RSV lacks HN proteins. RSV has two major

subtypes, A and B, which circulate concurrently in the population and are detected in similar ratios (54). Outbreaks with one subtype predominating can occur but vary depending on geographic range and year (55).

1.3.1.2.3 Metapneumovirus (MPV)

MPV is a recently discovered virus that has been implicated in human respiratory disease. MPV, unlike other viruses, was originally detected using reverse transcriptase polymerase chain reaction (RT-PCR) and assigned to the metapneumovirus genera based on clinical data, sequence homology and gene alignment (30). MPV is a large (200 nm diameter), non-segmented, enveloped, helical virus. Genetically it is similar to avian pneumovirus, hence the name, metapneumovirus. Two major strains, A and B have been elucidated with further subtypes discovered (56). The genome is thought to contain eight genes that encode nine proteins (57, 58). Similar to RSV, it is predicted that the F and G glycoproteins of MPV are the major antigenic portions of the virion. Unlike RSV, MPV lacks non-structural proteins which counteract interferon-alpha induction/production in the host (59). What this means for the pathogenicity of MPV and the resultant host immune response remains unclear.

1.3.2 Single Stranded Positive Polarity RNA Viruses

The families of viruses included in this group are coronaviridae and picornaviridae (rhinovirus/enterovirus) which cause the majority of human upper respiratory tract infections (URTI). Since viral RNA is of positive polarity, it is able to function directly as mRNA and immediately start infecting and reproducing within its host.

1.3.2.1 Human Coronavirus (HCoV)

HCoV is a large (80-160 nm diameter), non-segmented, enveloped, helical virus (60). The family is named after the Latin word for crown due to the large widely spaced spikes on the surface of the virion when viewed by electron microscopy (61). The antigenic portion of the virus consists of a glycoprotein found on the envelope of the virus. For all HCoV, this is the spike (S) glycoprotein and is involved in receptor binding and cell fusion (60). The membrane (M) glycoprotein which is involved in transmembrane budding and envelope formation is mostly internal. Some HCoV, such as OC43, also have a third glycoprotein known as hemagglutinin-esterase (HE) and like the S glycoprotein, can be antigenic and neutralized by host antibodies (40).

1.3.2.2 Human Rhinovirus (HRV)

HRV is a small (27-30 nm diameter), non-segmented, non-enveloped, icosahedral virus (62). The capsid is comprised of 60 copies each of the four capsid proteins (VP1-VP4) of which the first three form the external surface (40). Under the electron microscope, the virus appears smooth and round. HRV consists of over one hundred different serotypes and only five of these have been fully sequenced (62). HRV can be grouped based on the attachment of the virus to host cell receptor. Over 90 serotypes bind to intercellular adhesion molecule-1 (ICAM-1) while the remaining serotypes bind to low density lipoprotein receptor (LDL-R) (63). The one exception is HRV 87 which appears to require a sialic acid moiety on the host cell to attach (64).

1.3.3 DNA Viruses

1.3.3.1 Adenovirus

Adenoviridae makes up a large component of this family of viruses comprising of six subgroups (A-F) and over 50 serotypes (65). Adenovirus is a double-stranded, medium-sized (70-80 nm diameter), non-segmented, non-enveloped, icosahedral virus (40). Antigenic portions of the virus include the penton and fibre proteins which have type-specific antigenic epitopes and hexon protein which has both type-specific and group-specific antigenic epitopes (66, 67). Adenovirus has many targets in the human body, including the respiratory tract, gastrointestinal tract and eye. Three subgroups of adenovirus are implicated in the majority of human respiratory infections: B, C and E (65). Other DNA viruses including those from the human herpes viral family will not be emphasized in this thesis because they are not common causes of respiratory infection unless in specialized settings such as immunocompromised patients or those with underlying chronic disease, in which herpes viruses can cause serious respiratory infections.

1.4 Epidemiology

1.4.1 Influenza Virus

Influenza is one of the most important emerging and re-emerging infectious diseases, having caused epidemics and pandemics for centuries and with high morbidity and mortality. Influenza has certain distinctive epidemiologic features. In temperate zones, the influenza season occurs during late fall and winter. Epidemics occur every few years. The incidence of clinical influenza illness, based on symptoms and observable signs (see section 1.4), can be as high as 40% in

children (68). In the United States alone, the number of influenza related deaths total between 20,000 to 40,000 per year (69). In terms of leading causes of death, mortality due to influenza, particularly influenza-associated pneumonia, ranks seventh overall in the United States as of 2002 (70). Schools and industry both display high rates of absenteeism and excess mortality is evident in the elderly and individuals with chronic health problems. The most well known influenza pandemic is the Spanish influenza of 1918 which caused approximately 20-50 million deaths worldwide (71). This strain was exceptionally lethal in that the morbidity and mortality rates in healthy adults, 20 to 40 years of age, were extremely high. The hallmark of the 1918 H1N1 strain was its ability to penetrate deep into the lung parenchyma, causing pneumonia (72).

1.4.2 Parainfluenza Virus

Epidemiological studies show that PIV generates disease year round; PIV-1 peaks during autumn, PIV-2 in winter and PIV-3 in spring/summer (52). PIV is thought to be only second to RSV in hospitalizations in children due to viral LRTI. PIV-3 achieves an infection rate of close to 50% in children by the age of one and by six years of age, virtually all children have been infected. PIV-1, -2, and -4 infect most children by the age of ten years (73, 74). PIV is estimated to cause 50 to 75% of cases of croup, 10 to 15% of cases of bronchiolitis, and 10% of cases of pneumonia in children (52, 75). Immunity to PIV infection is transient and re-infection occurs regularly. Persistent infections may develop in patients who are immunocompromised (76). PIV is a significant nosocomial pathogen in the pediatric wards. Control measures may be taken to minimize spread of infection and this may involve designating certain areas for those infants with croup. Other precautions that may be undertaken include ward closures, proper cleaning and

disinfection of affected areas, proper personal hygiene, proper personal protective clothing for staff, and minimizing or excluding visitors.

1.4.3 Respiratory Syncytial Virus

RSV infections peak during the winter months. RSV circulates globally and predictably generates a substantial outbreak every year as the infectious season runs from fall to late spring (77). Highly contagious, RSV is thought to infect nearly all children by the age of five years (78-80). RSV is the most common cause of severe LRTI in children and is estimated to cause approximately 50 to 90% of cases of acute bronchiolitis, 20 to 40% of cases of pneumonia and about 10% of cases of croup (80-82). Mortality rates are around 5% for hospitalized children and up to 40% for those hospitalized with underlying medical conditions (83). Overall mortality rate is approximately 1% (84). Hospital pediatric wards are a common source of infant RSV infections. Infected infants who are handled by hospital staff or infected staff who have mild symptoms may easily spread the virus to other infants by contact of the eyes and nose. Like PIV, immunity to RSV is temporary as an incomplete host immune response allows repeated infections to occur throughout life (85).

1.4.4 Metapneumovirus

Analyses conducted thus far have revealed that MPV can be found globally (39, 86-93). MPV strikes more heavily during the winter months in temperate climates like influenza. Peak infection rates occur during winter and early spring, which overlaps the RSV season. MPV infection tends to culminate during the latter half of the RSV season (93, 94). Results of serological studies indicate that most children have been exposed to MPV by the age of 5 years

and virtually all by the age of 10 years (30, 95). Overall, MPV has been found to account for 3 to 12% of "flu-like" illness (39, 91, 93, 96) . MPV, while only recently discovered, has been found to have existed in the human population for at least 50 years based on retrospective serological studies documenting the presence of antibodies against the virus (30).

1.4.5 Human Coronavirus

Very few epidemiological studies have been conducted into HCoV infections. What is known is that infection rates are found to be uniform across all age groups which is markedly different from many other respiratory viruses (which have a tendency to decrease as age increases) (97). Infections are greatest during the winter months and early spring (98). In any one year, the majority of coronaviral infections can be ascribed to one group of viruses and this interchanges from year to year (99). This pattern is found worldwide. Re-infections by HCoV of the same strain occur frequently as immunity has been found not to be permanent (100). Approximately 20 to 30% of the "common cold" is believed to be attributed to HCoV infection (101).

Coronaviruses infect not only humans, but mammals and birds as well. In late 2002, the Guangdong province of China had an outbreak of a new respiratory disease termed SARS, characterized by a high fever, headache, fatigue and myalgia with the development of a dry cough and dyspnea (102). Gastrointestinal symptoms such as diarrhea developed in close to 40% of patients (103). Respiratory distress eventually led to death in about 10% of those infected. By June 2003, the outbreak had subsided with 774 deaths out of approximately 8000 cases worldwide (104). Preliminary assessments were conducted on clinical specimens from patients infected with SARS. A partial sequence recovered from a PCR-based random-amplification

procedure placed the novel virus into the coronaviridae family; electron microscopy revealed structural features characteristic of HCoV and immunofluorescence showed reactivity to group I CoV antibodies (104, 105). Sequencing of the SARS-CoV genome was determined by Centers for Disease Control in Atlanta and the Genome Science Centre of the BC Cancer Agency (106, 107). It was originally believed that civet cats and a few other species of animals were natural reservoirs for SARS-CoV (108). Further research conducted ascertained that the Chinese horseshoe bat is the natural reservoir for SARS-CoV (109, 110). Like wild ducks and influenza, Chinese horseshoe bats are largely unaffected by SARS-CoV. Presumably, civet cats were infected by these bats and virus was shed through the cats' feces. It is thought that in the food markets where these animals are considered a delicacy, people became infected because they were in such close quarters with the diseased cats. In fact, approximately one third of original SARS cases were attributed to food handlers in these markets (108, 111).

1.4.6 Human Rhinovirus

HRV infection may occur year round with peaks in the fall and spring months. HRV has been implicated in up to 80% of all respiratory infections (112, 113) and around 90% of children under two years of age have had a rhinoviral infection (114). As there are over 100 known serotypes, re-infection with a different serotype occurs regularly. Infections are most common during childhood and commonly decline as age increases. This is mainly due to the presence of neutralizing antibodies against previously encountered serotypes. However, lifelong immunity is not necessarily acquired since antibody concentrations in the blood decline over time (63). Transmission of HRV is mainly through inhalation of aerosols (115). Close contact in a high density population also increases the chance of viral transmission.

1.4.7 Adenovirus

Adenoviral infections occur throughout the year. Adenovirus infection is endemic not only to the human population but also mammals, birds and amphibians (116). Approximately 80% of children by the age of five, has been infected by one or more subgroups of adenovirus (117). Approximately 2 to 10% of respiratory infections in children under the age of four can be attributed to adenovirus (118). Out of that, 10% are hospitalized, generally with croup or pneumonia. Adenoviral serotypes 1 through 7 are the most prevalent (118, 119) and virtually all adults will have antibodies against these 7. Once infected with a certain serotype, lifelong immunity is conferred. While neutralizing antibodies protect against re-infection with the same serotype, virus may be shed for months after the primary infection due to the host's carrier state (66). As with most respiratory viruses, the frequency of infection is higher in children than adults. Control and prevention include implementing more stringent hygienic strategies since adenovirus is a resilient, stable virus that can survive various environments and can spread easily through the population.

1.5 Clinical Manifestations and Therapy

Infection by many of the respiratory viruses share several of the same clinical consequences that are generally referred to as "flu-like" or "cold-like" illness. These can include but are not limited to: fever, sore throat, sneezing, chills, headache, myalgia, coryza, rhinorrhea, fatigue, nasal congestion and cough. Most viral infections generate mild and self-limited clinical disease; however, infants, the elderly, those with underlying chronic or medical conditions, and immunocompromised individuals may develop more severe and prolonged disease.

Transmission of these pathogens tends to be identical as most are spread through person-to-person contact through respiratory aerosols produced by coughing, sneezing and speaking. The viruses may also be spread by contaminated fomites or through contact from hands to the nose and/or eyes. Distribution of infection may be restricted by observing good hygienic practices.

1.5.1 Influenza Virus

Concerning influenza viruses, the incubation period lasts from one to three days while the duration of illness lasts about a week (120). Symptoms such as fatigue and cough may persist for an additional seven to fourteen days. Infants, elderly and immunocompromised patients are also prone to acquire a secondary infection, commonly bacterial pneumonia, which accounts for the high mortality rates seen with influenza infection (121). Children are apt to have higher fevers that may be accompanied by febrile convulsions and may develop otitis media and gastrointestinal difficulties such as diarrhea and abdominal pains (122).

Control measures include prophylaxis with vaccine, or prophylaxis and/or therapy by antiviral drugs. There are two types of influenza vaccines, inactivated and live-attenuated. Inactivated vaccine consists of three strains, two type A influenza (H1N1, H3N2) and one type B influenza. The strains incorporated into the vaccine represent the most recent antigenic variants. The viruses are grown in hen eggs and inactivated using formaldehyde or beta-propiolactone rendering them non-infectious (123). The vaccine is approximately 70 to 80% effective (124) depending on the age and immunocompetence of the recipient and the similarity between the viral strains in the vaccine and those circulating in the community. Live-attenuated vaccine (FluMist®) is given intranasally and its advantage over its inactivated counterpart is that it

mimics natural infection which allows for a broader immune response. It is a cold-adapted strain that grows well in the upper respiratory tract but poorly in the lower respiratory tract. FluMist® has been shown to be safe and approximately 90% effective (125).

Antiviral drugs are available for influenza virus prophylaxis and therapy. The key objective of these drugs is to inhibit viral replication, thereby reducing the number of infectious particles. Adamantane derivatives, amantadine (Symmetrel®) and rimantadine (Flumadine®) are effective for the prevention and treatment of type A influenza only while oseltamivir (Tamiflu®) and zanamivir (Relenza®) are effective for both type A and B influenza (41). Treatments must begin within forty-eight hours of the onset of symptoms to help diminish the severity and duration. The adamantane derivatives both prevent membrane fusion by binding and block the M2 ion channel protein (126) while oseltamivir and zanamivir are NA inhibitors (69). Treatment with either class of antiviral drug is not recommended for general influenza outbreaks. The main problem with both classes of antiviral drug, particularly the adamantane derivatives, is the ease of resistance the virus is able to form (127, 128). Antibodies against HA neutralize the infectivity of the influenza virus while antibodies against NA reduce the reproduction of the virus and severity of disease.

1.5.2 Parainfluenza Virus

The incubation period of PIV may last between two to six days (129). In addition to the general symptoms listed above, PIV infection may involve stridor (a high pitched sound heard during inhalation due to constricted airways), loss of voice and dyspnea. The hallmark clinical manifestation of PIV infection is croup (laryngotracheobronchitis) (75), which is caused by

strains 1 through 3. Types 1 and 2 can also cause pharyngitis, tracheobronchitis and the "common cold". URTI, bronchitis and bronchopneumonia tend to be associated with strains 1 and 3 (52). In fact, type 3 infections sometimes manifest clinically like a RSV infection in children with bronchiolitis and pneumonia as possible disease outcomes. Type 4 infection most often develops into mild URTI. Adults tend to only contract mild URTI with any type of PIV infection.

In the late 1960s, vaccines based on whole PIV-1, -2 and -3 that were formalin-inactivated (130, 131) were administered to children. The vaccines were ineffective as children developed antibodies against all three serotypes but at a level much lower than natural infection. Live-attenuated vaccines based on human and bovine strains are currently in development (132, 133).

1.5.3 Respiratory Syncytial Virus

RSV incubates for a period of three to six days (134). Initially, infants exhibit a febrile URTI with possible apnea as the infection takes hold in the respiratory epithelium (135). Over the course of a few days, involvement of the lower respiratory tract may become evident as the infant develops cough, dyspnea, and tachypnea and becomes hypoxemic (136). Bronchiolitis or pneumonia may follow. Otitis media is often found in conjunction with RSV infection (137). Resolution of illness is variable as it may last several weeks. Most infants are able to recover with no detectable sequelae. Those that develop a LRTI may be subject to protracted alterations in pulmonary function leading to possible chronic lung disease throughout life. Studies have shown children with respiratory sequelae due to RSV infection are prone to recurrent LRTI and wheezing (138-140). Infections in older infants generally result in URTI or tracheobronchitis

(135) and resolution of the URTI usually takes longer than the average cold. Re-infection by RSV throughout life is common due to the existence of strain variation (85). However, in older children and adults, infection tends to be restricted to the upper respiratory tract causing mild cold symptoms or bronchitis (135). The elderly are prone to more severe infection, such as pneumonia, as their immune systems are not as robust (141). RSV is also known as a "persistent" virus in that it continues to replicate at low-levels for a protracted amount of time long after acute symptoms have resolved. Studies of animal models (142, 143), cell cultures (144), and patients (145) suggest the ability of RSV to persist within the lung, avoiding detection and eradication by the host immune response. There is thought that this persistence may function as a reservoir of infection between outbreaks.

Management of RSV infection has varied. A formalin-inactivated vaccine was developed in the 1960s to prevent RSV infection in infants and children. However, it was soon observed that the vaccine did not prevent infections from occurring but was actually causing more severe disease in some children who were inoculated and subsequently contracted a RSV infection (131, 146). A number of live-attenuated vaccines followed but were rendered unusable as they caused too strong a host immune response or were unstable (147). Current research has gone into manufacturing subunit vaccines based on the envelope glycoproteins, F and G (148); recombinant vaccines based on deletion of non-structural (NS2); and small hydrophobic (SH) genes (149) and a F glycoprotein inserted into a Newcastle disease virus vector (150). Which components of the host's immune response involved in responding to RSV infection is not very well understood and therefore efforts to create an effective vaccine have been hindered. Virazole (Ribavirin®), an antiviral drug intended to interfere with mRNA expression, is the only drug

approved for RSV pharmacotherapy. However, studies conducted on ribavirin's toxicity and efficacy were found to be questionable and it has consequently fallen out of favour for therapeutic use (151, 152). Prophylactic treatments available include RSV-IGIV (RespiGam™) and palivizumab (Synagis®) (153). RespiGam contains a comprehensive spectrum of antibodies since it is produced from an amalgamation of high titre anti-RSV antibody from the plasma of a large number of normal, healthy individuals. It is targeted towards the F and G glycoproteins of RSV and clinical trials have established RespiGam to be effective in reducing the severity of infection, number of hospitalizations and time spent in hospital (154). Synagis consists of a chimeric human-murine IgG-kappa monoclonal antibody that is designed to selectively react with the A antigenic site of the F glycoprotein. Synagis is effective against both strains of RSV (155) since the F glycoprotein has been found to be highly conserved between the A and B strains.

1.5.4 Metapneumovirus

As MPV belongs to the same subfamily as RSV, it shares many of the same clinical attributes (156). Less frequent symptoms include wheezing, dyspnea, pneumonia, bronchitis, bronchiolitis, conjunctivitis and otitis media (157). While MPV generally manifests as a mild URTI, it can develop into bronchiolitis and pneumonitis in infants and bronchitis and pneumonitis in elderly and immunocompromised patients (158). Like RSV, tissue tropism is to the respiratory epithelium.

While studies have demonstrated universal infection of MPV, re-infections occur in adulthood suggesting that immunity may not be permanent. This could be due to the non-initiation of a

specific humoral response or it may be due to antigenic differences between different strains that may not be recognized by the antibodies already produced by a previous MPV infection (157, 158). There are currently no vaccines, antiviral agents or prophylactics available specifically for the prevention or treatment of MPV infection. Development of live-attenuated and recombinant viral vaccines are under investigation (159, 160). A recent study has shown *in vitro* therapy with ribavirin or intravenously administered immune globulin has a similar response in both RSV and MPV (161). This result suggests prospective usage of either antiviral for treatment of MPV infection. Tissue culture assays exposed to NMSO3, a sulfated sialyl lipid, and heparin have demonstrated replication inhibition of MPV (162).

1.5.5 Human Coronavirus

HCoV incubates between one to four days (163). Infection is localized within the epithelium of the nasopharynx and subsequently spreads throughout the upper respiratory tract. Infected cells show vacuolation and damaged cilia which triggers the production of inflammatory mediators and causes mild clinical symptoms which resolve within a few days. Some evidence shows that coronaviruses are responsible for a significant proportion of lower respiratory tract infection in infants and the elderly (97, 164). No antiviral drugs or vaccines are currently available for HCoV-229E or -OC43 infection; however, a recent study has established the ability of saikosaponins to significantly inhibit HCoV-229E attachment and penetration *in vitro* (165).

1.5.6 Human Rhinovirus

HRV is best known for causing the common cold and is the main cause of the majority of upper respiratory tract infections (113). However, infections with HRV tend to be mild in severity with

no lasting sequelae with the exception of children who have underlying chronic illnesses or compromised immune systems (166). The primary site of infection and damage caused is the nasal epithelium through binding to ICAM-1 and, to a lesser extent, LDL-R (63).

Incubation time is very short as viral titres are maximal by the second or third day of infection (167). By the fifth day, the virus is virtually undetectable though the duration of illness may last for up to two weeks (62). In young children, otitis media (114) and sinusitis (168) may occur, coinciding with a bacterial infection.

No vaccines are available for HRV due largely to the number of serotypes involved. Since infection from one strain does not confer immunity to another strain, prevention of infection by vaccination is improbable (63). Some research has focused on the development of antivirals which could be used for treatment and prophylaxis though there are no approved therapies. Pleconaril is used in treatment of picornaviral infections which blocks the attachment and uncoating function of the virus. A recent study found that pleconaril's efficacy was related to the susceptibility of the baseline virus isolate to the drug (169). Current clinical trials exhibit promising results with ruprintrivir and possibly pyridazinyl oxime ethers (170).

1.5.7 Adenovirus

Adenoviral incubation time varies widely, from 2 to 14 days (66). A characteristic feature of adenoviral infection, which occurs after the acute phase of infection has elapsed, is the establishment of a latent infection in the lung (171), tonsils (172), adenoids and lymphoid tissue (173) where the virus replicates at a low level for months thereafter. Adenovirus is a common

cause of bronchiolitis in children and adolescents (118, 174). Sequelae include bronchiectasis and abnormal lung function (175). Certain serotypes from subgroup C have been found to persist in tonsils for years (176). There are a number of ways that the virus is thought to persist. It may be found in lymphocytes in episomal form, integrate into the host DNA, or replicate at a low level such that it avoids detection by the host's immune system (177). Re-activation of the virus occurs through various physical and physiological factors including changes in host immunity levels or susceptibility of cells.

New military recruits have a tendency to contract pneumonia (178). Common strains involved include 4 (E), 7 (B) and 21(B) (179). Overcrowding can exacerbate the severity of symptoms as recruits are continually exposed to high viral titres.

Antiviral treatments for respiratory adenoviral infections have not been manufactured. Ribavirin and cidofovir were used for treatment at one time in immunosupressed patients but recent studies questioned the efficacy of the drugs and they have since fallen out of favour (180-182). A live non-attenuated vaccine incorporating types 4 and 7 and sometimes type 21 has been developed for use in the military and found to be safe and effective (183). However, manufacturing of the vaccine was discontinued as the military and sole maker of the vaccine were unable to come to an agreement to continue production (184). It was given orally as an enteric coated capsule bypassing the respiratory epithelium and replicating within the gut. This allowed for mild asymptomatic infection yet provided the immunological response needed to prevent more severe respiratory infections from occurring.

1.6 Summary

Each of these respiratory viruses has a global impact, affecting millions of individuals annually. **Table 1** summarizes and compares the respiratory viruses. All of the viruses can share various clinical symptoms and therefore it is difficult to differentiate for a specific virus on that basis alone. Detection techniques have only recently improved to facilitate more rapid diagnosis yet treatment for the most part is rudimentary. For a variety of reasons, including the diversity of viral life cycles, host cell receptors involved, number, structure, and function of viral genes and gene products, it is difficult to produce and generate any sort of generally effective anti-viral therapies and vaccines. Further investigation and experimentation from all aspects in this field are needed, which can be facilitated by improvements in viral detection. Chapter 2 will describe in more detail many of the methods used for diagnosis of human respiratory viral infections.

Table 1. Summary and comparison of respiratory viruses.

Virus	Structure	Replicates in	Cellular Receptors	Approved Antivirals	Approved Vaccines	Immunity
Influenza A	ss(-)RNA, enveloped, helical, segmented genome	nucleus	Sialic acid linked to galactose	amantadine, rimantadine, oseltamivir (Tamiflu), zanamivir	inactivated flu shot (2 strains type A, 1 strain B), Flumist	transient due to genomic antigenic drift and shift
Influenza B	ss(-)RNA, enveloped, helical, segmented genome	nucleus	Sialic acid linked to galactose	oseltamivir (Tamiflu), zanamivir	inactivated flu shot (2 strains type A, 1 strain B), Flumist	transient
PIV-1	ss(-)RNA, enveloped, helical, non-segmented genome	cytoplasm	possibly a sialic acid conjugate	none	none	transient
PIV-2	ss(-)RNA, enveloped, helical, non-segmented genome	cytoplasm	possibly a sialic acid conjugate	none	none	transient
PIV-3	ss(-)RNA, enveloped, helical, non-segmented genome	cytoplasm	possibly a sialic acid conjugate	none	none	transient
RSV A	ss(-)RNA, enveloped, helical, non-segmented genome	cytoplasm	unknown	ribavirin, RespiGam, Synagis	none	transient
RSV B	ss(-)RNA, enveloped, helical, non-segmented genome	cytoplasm	unknown	ribavirin, RespiGam, Synagis	none	transient
MPV	ss(-)RNA, enveloped, helical, non-segmented genome	cytoplasm	ACE2	none	none	transient
hCV 229E	ss(+)RNA, enveloped, helical, non-segmented genome	cytoplasm	hAPN (CD13)	none	none	transient
hCV OC43	ss(+)RNA, enveloped, helical, non-segmented genome	cytoplasm	affinity for HLA I and 9-O-aceytlated sialic acid receptor	none	none	transient
HRV	ss(+)RNA, non-enveloped, icosahedral, non-segmented genome	cytoplasm	ICAM-1, LDL-R	pleconaril	none	permanent until circulating Ab levels drop
Adenovirus	dsDNA, non-enveloped, icosahedral, non-segmented genome	nucleus	CAR, MHC-I alpha 2, CD46, sialic acid containing receptor	ribavirin, cidofovir	live non-attenuated enteric coated capsule (military use only)	permanent

2. VIRAL DIAGNOSTIC TECHNIQUES

2.1 Introduction

A number of diagnostic techniques currently exists for accurate identification of a viral infection. This allows for viral diagnosis for the individual patient, monitoring for outbreaks of seasonal viruses and contributes to physicians' and clinical scientists' understanding of a disease. It also helps advance epidemiological and public health studies which in turn facilitates improved disease control and prevention and community health and education. Viral diagnosis is accomplished through viral isolation and identification through subtyping, various serological techniques, or detection of viral proteins or nucleic acids. Each of these tests focuses on different aspects of virus and host response and as a result, there is no comprehensive test that encompasses both. Each test will be discussed in further detail below. Rapid verification is desirable to initiate proper infection control and appropriate anti-viral therapy for the patient, if available.

Nasopharyngeal aspirates (NPAs) remain the best source of specimens for diagnostics mainly because the tissue tropism for the majority of common respiratory viruses is the epithelium of the nasopharynx. Use of nasopharyngeal swabs as test specimens for viral culture has shown a 30% reduction in detection sensitivity compared to NPAs (185, 186). Sputum has been found to produce comparable results as NPAs and may in fact be more representative of the respiratory tract as NPA samples exclusively from the upper respiratory tract (187). However, acquiring induced sputum from patients can prove to be difficult. Bronchoalveolar lavage may also be used (188, 189) but is more invasive and may not be easy to obtain from patients.

2.2 Culture

Viral isolation, through *in vitro* approaches, is the usual "gold standard" of laboratory diagnosis of respiratory viral infections which tests for viral replication. Many respiratory viruses will grow in various cell or tissue culture lines. Primary cell lines (e.g., monkey kidney (MK)) are obtained through freshly killed animals and may only be passaged once or twice. While they are considered to be superior cell lines as they are permissive to many viruses, they are costly and obtaining a consistent source can be problematic. Cell lines (e.g., transformed human embryonic kidney, HEK-293, MRC-5) obtained from embryonic tissue may only be passaged up to 50 times as the cells eventually lose their ability to divide, or senesce. They are the easiest to handle but each line is permissive to a limited number of viruses. Continuous cell lines (e.g., HeLa, Vero, HEp-2, LLC-MK2) are derived from malignant tumours and are termed "immortalized" cells because they may be passaged indefinitely. The test specimen is added to permissive cell lines and left to incubate at a constant temperature in a rotating drum or incubation cabinet. Growth media may be changed daily to supplement the cells with nutrients. The cells should be examined frequently for the presence of cytopathic effect (CPE). Initial identification of viral isolates may be detected through CPE or hemadsorption (cell surface changes) while confirmatory identification may use immunofluorescence (IF), neutralization, hemagglutinin-inhibition, viral antigen detection or molecular testing. The main limitation of viral isolation is that obtaining results may necessitate a lengthy incubation time ranging from a few days to a few weeks, and some viruses may not replicate *in vitro* (190). Culture sensitivity can be poor as a specimen with intact, viable virus is required for positive results while contamination of cell lines, maintenance of cell lines or the presence of inhibitory substances add to the difficultly of isolating viruses.

Influenza may be cultivated in primary rhesus monkey kidney (PMK), LLC-MK2, A549, and Madin Darby canine kidney (MDCK) cells (191). Type B influenza will produce a cytopathic effect (CPE) when grown in MDCK cells. Presence of type A influenza is further distinguished using immunostaining or a hemagglutinin-inhibition assay (HAI) as described in sections 2.2 and 2.3.2 respectively. PIV is commonly detected by viral culture using PMK, Vero, HEp-2, MDCK, HeLa or trypsinized LLC-MK2 cells (52). CPE occasionally occurs in PIV-2 and -3, producing large, multinucleated syncytia. HEp-2 cells are commonly used for isolating RSV, however many cells are permissible to RSV infection including HeLa, Vero, MRC-5 and WI-38 cells (53). RSV propagated in HEp-2, HeLa and MRC-5 cells exhibit a characteristic CPE of syncytial formation in approximately five to seven days (192). The ability to isolate MPV from cell culture has proven to be rather difficult as only tertiary MK, as well as LLC-MK2, HEp-2 and some Vero cells have demonstrated the capability to do so (30, 158, 193). Compounding this challenge is that CPE takes approximately two to three weeks to develop. CPE has been found to be variable as some show RSV-like syncytia and others cell rounding (158). Coronaviruses are fastidious and will only grow in human embryonic fibroblasts, suckling mouse brain, or organ cultured cells (194). Syncytia may form. Generally, HRV is detected by viral culture using human embryo lung fibroblasts like MRC-5 and WI-38, or HeLa cells (195). CPE consists of highly refractile cell rounding which is very similar to enteroviral CPE. HRV can be differentiated from enteroviruses since HRV is acid-labile with many research laboratories using this technique to distinguish between the two. A number of cells may be used for viral isolation of adenovirus. HEK-293 cells are permissive for all respiratory strains of adenovirus (65). Other cells that the virus may be propagated in include: A549, HEp-2, HeLa, primary MK, MRC-5, SF, WI-38 and KB (66). CPE occurs anywhere from two to seven days after infection. The

characteristic CPE of adenovirus consist of enlarged, refractile, rounded cells that may be clustered.

2.3 Immunostaining

Antigen detection assays may involve immunostaining through direct or indirect immunofluorescence (IF). IF assays involve detecting viral antigens or virus specific antibodies (i.e., IgG, IgA, IgM) and visualizing the fluorescent cells under ultraviolet light. It is currently the most common method used for initial respiratory viral detection from patient specimens. Briefly, the direct method involves analyzing the test specimen with a primary or detection antibody that is labeled with a fluorochrome (e.g., fluorescein) against a specific viral antigen. The indirect method involves analyzing the test specimen with a non-labeled primary antibody followed by a fluorochome tagged secondary antibody. Viral antigen detection may be conducted with either direct or indirect IF whereas antibody detection is mostly carried out using the indirect method. Cells containing viral protein recognized by the primary antibody will produce a bright green stain. Cytoplasmic staining is normally speckled with large inclusions while nuclear staining is uniform. The test is rapid and simple; however the quality of the patient specimen is key since an insufficient number of cells will yield uninterpretable findings. Sensitivity of the technique varies between viruses. IF can become labour intensive, in particular, indirect IF since it entails an extra antibody step. Highly trained technicians are also required for assay interpretation.

Influenza, PIV, RSV and adenovirus may all be detected using IF (196). Detection of influenza and PIV antigen is generally achieved by direct IF. Detection of RSV using direct and indirect IF

may be conducted using poly- or monoclonal antibodies. Sensitivities for these assays range from 40 to 90%. Sensitivity of detection for RSV by IF is comparable to viral isolation. IF alone for adenoviral verification, using an anti-hexon monoclonal antibody, is not particularly sensitive. A direct IF assay for MPV was developed by generating monoclonal antibodies for detection of antigen in patient NPA, however it is mostly used for retrospective analysis at this time (197).

2.4 Serology

2.4.1 Complement Fixation Test

Complement fixation test (CFT) uses two-fold serial dilutions of paired sera from the acute and convalescent phases of infection, essentially testing for antibody production by the host's immune system. This assay consists of two reactions; the first involves the binding and inactivation, hence 'fixation' of an antigen-antibody complex in the presence of a known amount of complement, the indicator system. The second reaction involves hemolysing sensitized sheep erythrocytes coated with anti-sheep antibody. If hemolysis occurs, free complement is present, the antigen-antibody complexes did not develop and a 'negative' outcome results. If hemolysis does not occur, free complement is fixed by the antigen-antibody complexes indicating that the patient possessed antibodies against the test virus. A four-fold or greater rise in titre of total antibody is considered as indicating current infection (198). CFT has the ability to screen against a large number of viral infections simultaneously and is relatively inexpensive. However it is not particularly sensitive and it is time consuming and cumbersome with the time to acquire acute and convalescent sera taking days or weeks.

CFT may be used for new influenza viral subtypes whereas hemagglutinin-inhibition (HAI) is subtype-specific. CFT uses complement-fixing antibodies against test sera; however, results take longer than HAI and is therefore less utilized. At times it may be non-specific, as in the case of differentiating between PIV subtypes as cross-reactivity occurs and CF antibody does not develop on initial infection from some young children (52). CFT has not been found to be particularly sensitive or useful for RSV identification since seroconversion does not occur for at least a two week period (199). In young infants, the serological response can be quite poor and undetectable by some assays. CFT is the test of choice for adenovirus in diagnostic laboratories, however infants often do not respond with CF antibody after infection. Overall, sensitivity of the CFT assay has been found to be approximately 75% (200).

2.4.2 Hemagglutinin-Inhibition Test

Hemagglutinin-inhibition (HAI or HI) tests involve the ability of a virus to clump together or 'agglutinate' mammalian or avian erythrocytes. Respiratory viruses that are able to hemagglutinate include influenza, PIV and adenovirus. Like CFT, acute and convalescent sera from the patient are used. Each well in a microtitre plate is prepared with a known amount of virus and allowed to react to two-fold serial diluted sera, followed by the addition of erythrocytes. If virus specific antibody is present in the sera, the virus will be bound and the erythrocytes will not agglutinate, hence hemagglutinin-inhibition. If no antibody is present, the virus remains unbound and will cause the erythrocytes to clump. The highest dilution of antigen that allows for erythrocytes to completely hemagglutinate is defined as a HA antibody unit. A four-fold or greater rise in antibody titre or seroconversion between the paired sera indicates

recent infection (201). The test is simple, inexpensive, and more sensitive than CFT. However, the usefulness of HAI has limitations in terms of patient care since acquiring acute and convalescent sera takes days to weeks and cross-reactivity may occur for certain viruses such as the different strains and subtypes of influenza and PIV (198, 202).

Like RSV, MPV does not display the ability to agglutinate human or mammalian erythrocytes. Some strains of influenza react poorly to HAI testing and subsequently is not the first test of choice (203). As with CF, subtyping of PIV by HAI is not used due to cross-reactivity (52). Further typing by HAI is generally performed for adenovirus after viral culturing. HAI with monkey (subgroup B), or rat (subgroups A to F excluding B) erythrocytes may be used for typing (66).

2.4.3 Hemadsorption Test

Hemadsorption involves the ability of virally infected cells to adhere to erythrocytes. It is generally used in detecting influenza or PIV. For PIV subtyping, this test carries the greatest specificity of all the serological tests available (16). Both family of viruses contain hemagglutinin as part of their viral structure and therefore are able to adsorb guinea pig erythrocytes. The virus inoculated cell culture is added to the erythrocytes, allowed to incubate and then examined for the presence of hemadsorption under a microscope.

2.4.4 Neutralization Test

Virus or serum neutralization tests involve the loss of viral infectivity due to interactions between the virus and a specific antibody forming antigen-antibody complexes. This is due to the

antibody interference at various points in viral entry or release. The antibody may neutralize the virus by interfering with viral attachment to the host cell receptor or it may bind the viral capsid disabling the virus' ability to uncoat and release nucleic acid. Virus and test serum are combined and inoculated into the test system. Un-neutralized virus is then detected through CPE, hemadsorption, HAI, or plaque formation. HRV may be detected using neutralization tests. Serum neutralization tests, while the most cumbersome, are the most sensitive and type-specific adenovirus tests available with sensitivities approaching 90% (15).

2.4.5 Enzyme Linked Immunosorbent Assay

Enzyme linked immunosorbent assays (ELISAs) may also be used for virus detection with a number of commercial kits available. The basic principle of an ELISA is the detection of certain antibodies or antigens against the virus in sera or plasma. Influenza, RSV, PIV, HCoV, HRV and adenovirus may all be detected by ELISA. ELISA sensitivity tends to be high but is comparable to other serological tests that are cheaper. For some viruses, like RSV, sensitivity can be poor with ambiguous results which necessitate further testing for confirmation.

2.5 Conventional Polymerase Chain Reaction

Polymerase chain reaction (PCR) serves to amplify a specific portion of a gene, if present, many times over. Since both strands are copied each cycle, there is an exponential increase in the number of copies of the template. The main advantage of this technique is that it can detect very low amounts of template. After an initial incubation step to stimulate the heat-activated DNA polymerase to function, three major steps are involved: denaturation, annealing and extension. Denaturation allows the double stranded DNA template to melt into single strands. Annealing

temperature permits the primers to bind to specific sequences of the single stranded DNA. Extension permits the polymerase to produce a complementary copy of the template in a 5' to 3' manner from the 3' end of the annealed primer. These three steps are generally repeated up to 40 cycles. The products are then visualized on an ethidium bromide stained agarose gel. The main problem with conventional PCR is contamination as even small contaminants will be amplified. As well, this test will only give a 'positive' or 'negative' result with no indication of actual amount of template present.

2.6 Quantitative Real-Time Polymerase Chain Reaction

Current techniques in use for the detection of pathogens include culture, immunostaining, various serological assays like CFT, HAI, hemadsorption, neutralization tests and ELISA, and conventional PCR. Out of all of these, PCR can detect very small amounts of viral nucleic acids, assuming an intact starting template. Conventional PCR employs end-point detection for analysis of whether the target amplicon is present. It occurs during the plateau phase of the reaction and becomes apparent once PCR thermocycling and post-PCR processing has concluded. A newer technique has been developed combining traditional PCR technology with automated detection and quantification of a fluorescent reporter, known as quantitative real-time PCR (qPCR). The amount of fluorescence is directly proportional to the amount of PCR product in a reaction. qPCR confers many advantages over conventional PCR. For example, qPCR requires far less template, allows the user to monitor the reaction during the exponential phase of the reaction or 'real-time' which obviates the need for post-PCR electrophoretic gel analysis, which in turns reduces the possibility of cross-contamination.

Two types of PCR may be performed, one-step or two-step. One-step PCR combines the reverse transcription of RNA to cDNA and subsequent amplification of that cDNA, all into a one tube reaction with the required reagents and enzymes. Two-step PCR is performed in two individual reactions; reverse transcription of RNA to cDNA and then amplification of an aliquot of that cDNA. Some investigators consider two-step qPCR to be advantageous over its one-step counterpart since it is generally more reproducible and sensitive (204, 205). In addition, one-step qPCR has been characterized by a large accumulation of primer dimers that can interfere with specific amplification of the target sequence (206). As an additional integrity check, two-step qPCR also uses cDNA as opposed to RNA which is prone to degradation. In cases where the amount of specimen is minute, the production of cDNA helps to increase the number of possible assay reactions (207). The small amplicon size allows for improved amplification efficiency, the dynamic range of detection is increased, and less nucleic acid is required in the reaction. To increase sensitivity, a small DNA oligonucleotide specific for the template is used as a probe.

The probe consists of a fluorescent reporter dye, 6-carboxyfluorescein (FAM), located at the 5'-end and a quencher dye, 6-carboxytetramethylrhodamine (TAMRA), located at the 3'end. Each qPCR reaction utilized a FAM-TAMRA combination as they were individual reactions and not multiplexed. Multiplex PCR allows for the simultaneous amplification of several targets of interest in a reaction by using multiple pairs of primers (208). Multiplex is useful in the case of limited starting material and allows for considerable savings of time, effort and money. However, multiplex assays are difficult to engineer since each reaction must be thoroughly optimized with lower sensitivity than individual PCR and the preferential amplification of certain targets, non-specific targets or quasi-species posing additional obstacles (209). Because of these

potential issues, the studies presented in this thesis have focused on development of a panel of single target reactions. The probes are all highly specific; a mismatch of two or more base pairs is enough to prevent the probe from binding to the target. If the target is present, the probe anneals between the primer sites. The 5' nuclease activity of the AmpliTaq Gold DNA Polymerase cleaves the bound probe, separating the reporter dye from the quencher dye, during the PCR process. Released from the quenching effect of the 3' dye, the reporter is then able to fluoresce. The increase in fluorescence after each cycle and therefore the accumulation of PCR target amplicon can be monitored and detected by the thermocycler.

The reaction contains a passive internal fluorescent reference dye, 6-carboxy-X-rhodamine (ROX), which is pre-incorporated into the 2X Taqman Master Mix (Applied Biosystems, Foster City, CA, USA). ROX serves to normalize the target signal due to non-PCR related fluorescent fluctuations in reagent concentrations or total volume which may occur between samples over time. This is automatically calculated by the accompanying software. This differs from the normalization that is required to correct for PCR efficiency or amount of template used.

2.7 Summary

This chapter has provided an overview of some of the laboratory techniques commonly used for viral diagnosis, noting their various strengths and limitations. Overall, there is a need for a single technique that contains all these strengths in a rapid and easy-to-do format. Chapter 3 explains the reasoning and justification behind the developmental stage of the real-time quantitative PCR panel for respiratory virus detection.

3. RESEARCH OVERVIEW

3.1 Rationale

It is well established that traditional viral diagnostic techniques tend to be time-consuming, cumbersome or not particularly sensitive. Even conventional PCR suffers from various drawbacks as mentioned earlier in this thesis. Current qPCR protocols for common respiratory viruses also show variability in the pre-analytical and analytical phases, which can limit the successful translation to the clinical diagnostic laboratory setting (210). Consequently, many of these traditional techniques are still in use as there is no standard protocol or set criterion for assay interpretation for qPCR assays. The goal of this thesis was to develop a qPCR panel that is rapid, quantitative, and standardized, with the long-term goal to facilitate validation and ultimately use in the clinical laboratory for improved decision-making and intervention.

In addition, extensive experience in the area of HIV infection has clearly shown that disease activity, response to therapy, and progression are related to viral load (211, 212). It is possible that quantification of viral nucleic acid load in clinical respiratory specimens may also provide more information than merely identifying the presence of viral nucleic acid in specimens. The introduction of qPCR technology has facilitated the ability to reliably quantify target nucleic acid sequences.

The **central hypothesis** of this project is that **quantification of viral load in clinical respiratory specimens can allow for improved interpretations of viral PCR results in terms of understanding the relationship between viral nucleic acids detected by qPCR, viruses**

documented by other laboratory methods of viral diagnosis, and concomitant clinical features.

3.2 Specific Aims

The research was divided into a number of components:

1. Designing and developing individual quantitative real-time PCR assays for influenza A, influenza B, PIV-1, PIV-2, PIV-3, RSV A, RSV B, MPV, HCoV-229E, HCoV-OC43, HRV, and adenovirus using standardized conditions.

2. Designing a quantitative real-time PCR for a reference gene, hypoxanthine phosphoribosyl transferase (HPRT), that is suitable for normalizing gene expression in the test specimens.

3. Manufacturing synthetic standards for each of the respiratory viruses to allow for more accurate quantification for use in quality assurance.

4. Examine the relationship/concordance of qPCR results from archival NPAs to other laboratory techniques, specifically viral culture on the same samples.

5. Using qPCR results on archival NPAs to determine a possible "threshold" viral load level that is associated with a particular specimen exhibiting a positive culture result.

6. Performing a retrospective analysis using qPCR results from archival NPAs of the relationship between viral load and clinical respiratory illness diagnosed at the time of patient presentation.

After designing individual qPCR assays for each respiratory virus, archival NPAs obtained from symptomatic children at the time of emergency room visits were used to test the qPCR panel. Inclusion of a reference gene assay verifies that each specimen contains viral nucleic acid and permits normalization of results. Synthetic controls test the robustness of the qPCR assays and allow for the quantification of viral loads. One goal of this study was to better understand the differences between viral loads and corresponding culture results as well as viral loads and clinical diagnosis. If the paradigm of HIV viral load is also applicable to common respiratory viruses, then quantification may prove to be clinically important in terms of evaluation and treatment.

4. METHODS AND MATERIALS

4.1 Case Selection of Nasopharyngeal Aspirates (NPAs)

Archived NPAs from the 2003-2004 winter season, stored frozen at -80°C, were selected based on previously diagnosed laboratory results (culture and/or direct fluorescent antibody test). In total, 365 specimens were chosen (30 each positive for influenza types A and B, 40 each positive for PIV-1, -2, -3, RSV, adenovirus, 5 for HRV, 100 culture negative) for analysis. RSV subtypes were not discriminated for. A subset of 187 specimens from children was then selected for further examination based on availability of clinical diagnosis as noted on laboratory requisition forms. The first subgroup consisted of children with URTI (n = 23), a second contained children who had fever (n= 98) and a third contained children who were diagnosed with LRTI (croup, bronchiolitis, or pneumonia) (n = 66).

4.2 Nucleic Acid Extraction from Nasopharyngeal Aspirates

RNA from archival NPAs was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Mississauga, ON, Canada) following the manufacturer's instructions. One notable change from the protocol was the elution of the RNA from the standard 60 μL to 50 μL to concentrate the nucleic acid. 310 μL of carrier RNA (1 μg/μL) was dissolved into 31 mL Buffer AVL and aliquoted 560 μL per 1.5 mL microcentrifuge tube. Carrier RNA helped the viral RNA to bind to the QIAamp mini membrane, particularly if the sample was of low-titre. Briefly, 140 μL of NPA underwent lysis in the pre-aliquoted Buffer AVL which contained denaturing guanidine isothiocyanate (GITC) inactivating RNases. Ethanol was added to allow the RNA to bind to the silica gel-based membrane. The pH and high salt buffers ensured that protein and other

contaminants did not bind to the membrane. After washing with two buffers, AW1 and AW2, to remove any remaining contaminants and inhibitors, the RNA was eluted using Buffer AVE. Buffer AVE contains sodium azide which prevented microbial growth and subsequent contamination with RNases. The RNA was aliquoted and stored at -80°C until use.

DNA from archival NPA was extracted using the QIAamp DNA Mini Kit (Qiagen, Mississauga, ON, Canada) following the manufacturer's instructions. In a 1.5 mL microcentrifuge tube, 20 µL of proteinase K, 200 µL of NPA and 200 µL of lysis buffer were mixed together and allowed to incubate for 10 minutes at 56°C. 200 µL of anhydrous ethanol was added to the sample and the resultant admixture was then loaded onto a QIAamp spin column which underwent centrifugation at 12,000 x *g*, which allowed the DNA to bind to the column membrane. The lysate buffering conditions allowed optimal binding of DNA to the QIAamp membrane. Any contaminants and inhibitors were washed away with the supplied wash buffers AW1 and AW2 and the DNA was subsequently eluted in 100 µL of Buffer AE (10 mM TrisCl; 0.5 mM EDTA, pH 9.0). The DNA was aliquoted and stored at -80°C.

4.3 Selection of Viruses for Inclusion in qPCR Panel

The following 12 respiratory viruses were chosen for inclusion into the qPCR panel as they, collectively, form a large burden of disease in the general population (213, 214). These viruses include influenza types A and B, PIV-1, -2, and -3, RSV A, RSV B, MPV, HCoV-229E and -OC43, HRV and adenovirus. Both PIV and RSV have a limited number of strains; therefore, individual assays for each serotype were developed. In contrast, assays for HRV and adenovirus

were developed to provide a broad screen since there are a large number of strains known for each virus.

4.4 Design of Primer and Probe Sequences for Quantitative Real-Time PCR

Literature searches were conducted for published qPCR protocols for the respiratory viruses under study. In late 2003, these searches showed a limited number of publications reporting working two-step standardized qPCR protocols using the ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Published protocols for RSV A and B, MPV, influenza B and adenovirus were modified to complement the Taqman™ probe (Applied Biosystems, Foster City, CA, USA) detection system (65, 215-217). The original amplicon for RSV A was lengthened to include the sequence of a Cla I site that was inserted within the synthetic plasmid. The assays for RSV A and B were modified from a one-step qRT-PCR to a two-step qPCR. The original MPV assay used by Children's and Women's Hospital based on the Mackay paper (217) comprised of a 68 base pair amplicon which was too small to design an accompanying probe and did not include a Cla I site for discrimination between native and synthetic amplified sequences. A new MPV forward primer was slightly modified from the published sequence. The reaction mixture volume and cycling conditions for all of these previously published assays varied but were standardized to one set of parameters (see section 4.8). The influenza A and HRV assay protocols were kindly provided by Dr. Stephen Lindstrom from CDC Atlanta and Dr. Sebastian Johnston from Imperial College, London, England, respectively and adapted to the set standardized conditions of the other assays. Primers and probes for the remaining viruses (PIV-1, -2, -3, HCoV-229E, -OC43) were constructed by

myself with input from Dr. Nicholas Au, one of Dr. Rusung Tan's former trainees at Children's and Women's Hospital of British Columbia, Vancouver, Canada.

Sequences for the primers and probes for each respiratory virus were chosen using Primer Express™ software (Applied Biosystems, Foster City, CA) following recommended criteria. Amplicons were kept under 250 base pairs to allow for rapid amplification, G-C content kept between 20-80%, and runs of identical nucleotides were avoided. A 5' G on the probe was avoided as it causes quenching of the reporter dye even after cleavage. For the reference gene hypoxanthine phophoribosyl transferase (HPRT), the amplicon was designed to span an intron which prevented the amplification of pseudogenes. Each set of primers and probe were "blasted" against the NCBI nucleotide database to ensure specificity to its particular virus. **Table 2** shows the accession numbers for each virus, forward and reverse primer sequences, probe sequence, gene segment chosen, and size of amplicon fragment amplified.

The fluorescent probes were designed to anneal to a sequence internal to the PCR primers. Each probe was labelled with FAM (6-carboxyfluorescein) and TAMRA (6-carboxy-tetramethyl-rhodamine) on the 5' and 3' ends, respectively. The melting temperature of the probe is generally 10°C higher than the primers to avoid the extension of the primer when the probe has not yet bound. The Taqman probe is based on what is known as fluorescent resonance energy transfer (FRET) for quantification. When the probe is intact, the proximity of the reporter dye to the quencher dye inhibits any fluorescent signal. During the annealing phase of PCR, if the target sequence is present, the probe will bind to the template. When the 5' nuclease activity of the Taq DNA polymerase cleaves the fluorophore from the probe on the target strand, the two dyes are

separated and FRET ceases. Since the reporter dye is no longer being quenched, it emits fluorescence and this signal is measured by the plate reader on the machine. The amount of fluorescence is directly proportional to the amount of target amplicon present in the assay. Since the probe is only cleaved in the presence of the target sequence, it ensures that any non-specific amplification is not detected.

4.5 Host Reference Gene Selection

The choice of human reference genes is critical, since the selection of an unsuitable gene will lead to misinterpretation and skewing of any real changes in gene expression. Requirements for an optimal reference gene is based on what the gene is NOT: in particular, the gene should not be regulated, not be highly expressed, and does not have pseudogenes. HPRT has been found to be stable, does not have pseudogenes, and is expressed at much lower levels than the traditional reference genes like glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or beta-actin, making it much more suitable for normalization of gene expression over a wide range of transcript levels (218).

4.6 Standards for Quantitative Real-Time PCR

Synthetic standards were produced for each virus to allow absolute quantification of cDNA from the NPA specimens. The standards were comprised of bacterial plasmids which incorporate the target amplicon of each virus. This was advantageous for a number of reasons: it allowed for the validation of novel assays, contained a known sequence which allowed for accurate quantification since it was made up of only the pure species, a restriction site integrated within the amplicon allowed for the detection of any cross-contamination between the clinical specimen

and the synthetic one, and ease in preparation permits a virtually unlimited supply. Briefly, an RNA molecule with identical priming sites and amplification attributes of the real viral target was created from synthetic oligonucleotides. This RNA molecule had a unique feature in that it integrated a 6 nucleotide polymorphism within the target sequence to assist in discerning between genuine and manufactured amplicons in cases of possible template contamination. Dr. John Brunstein of Children's and Women's Hospital of British Columbia kindly constructed and provided synthetic standards for RSV A and B, MPV, HCoV-229E and -OC43, and PIV-1. The design and assembly of synthetic standards for influenza A and B, PIV-2, PIV-3, HRV, adenovirus, were done in-house. **Figure 1** is a cartoon representation of a general synthetic control describing the viral gene insert, its orientation, restriction enzymes involved and the vector used.

4.6.1 Annealing, Ligation and Transformation

Based on the length of the amplicon, an average of six oligonucleotides (three forward and three reverse which were complementary) which included a Cla I restriction site were synthesized (Sigma Genosys, Oakville, ON, CAN) and phosphorylated using T4 Polynucleotide Kinase (Invitrogen, Burlington, ON, CAN). The T4 Kinase inserted a phosphate group to a 5' hydroxyl group on the oligonucleotide which is required for ligation. A 25 µL reaction volume consisting of 10 µL (15 mM) oligonucleotide, 5 µL of 5X Forward Reaction Buffer, 1 µL of T4 Kinase, 2.5 µL ATP and 6.5 µL water were combined together in a 0.5 mL microcentrifuge tube, vortexed briefly, pulse centrifuged and allowed to incubate at 37°C for 10 minutes. The reaction was terminated by incubating the mixture at 65°C for 10 minutes. To hybridize the oligonucleotides

prior to ligation, they were incubated using a step-wise decrease in temperature starting at 95°C and ending at 40°C for 1 minute each.

The oligonucleotides were ligated together using T4 DNA Ligase (Invitrogen, Burlington, ON, CAN) which catalyzed the formation of phosphodiester bonds between double-stranded DNAs with 3′ hydroxyl and 5′ phosphate termini in the presence of ATP. A reaction consisting of 9 μL oligonucleotides, 10 μL 2X ligase buffer, 1 μL 10 mM ATP and 1 μL T4 DNA ligase was left to incubate at 15°C overnight. The reaction was terminated by heating to 75°C for 10 minutes. The contents of the reaction were run on a 2% agarose gel to separate the ligated and unligated species. The band containing the ligated species was excised and gel purified using a MinElute Gel Extraction Kit (Qiagen, Mississauga, ON, Canada). Three gel volumes of buffer QG was added to the gel slice and incubated at 50°C for 10 minutes with vortexing of the mixture every 2-3 minutes during incubation. One gel volume of isopropanol was added and mixed by inverting the tube several times. The mixture was applied to a MinElute column and centrifuged at 12,000 x *g* for 1 minute. After the addition and centrifugation of 750 uL Buffer PE to wash and purify the oligonucleotide, 10 uL of Buffer EB was added to elute the oligonucleotide.

The purified ligated synthetic oligonucleotide was then cut with two restriction enzymes, Pst I on the 5' end and Xba I on the 3' end. The cloning vector, pGEM4Z (Promega, Madison, WI), was digested with the same two restriction enzymes which allowed the synthetic oligonucleotide and plasmid to ligate in a specific orientation. Using a 1:3 vector:insert ratio, a 10 μL reaction containing 100 ng of pGEM4Z vector, approximately 16 ng insert DNA, 1 unit of T4 DNA ligase, 1μL of 10X ligase buffer and water were combined in a 1.5mL Eppendorf tube and

allowed to ligate overnight at 16°C. The ligase was heat inactivated by incubating at 65°C for 10 minutes.

The recombinant vector was then heat-shocked with *E. coli* Stop Unwanted Rearrangement Events (SURE®) (Stratagene, La Jolla, CA) cells. Briefly, an aliquot of SURE® competent cells was thawed, and separated into 100 μL portions in pre-chilled polypropylene round-bottom tubes to which 1.7 μL of β-mercaptoethanol was added, blended by swirling gently before incubating on ice for 10 minutes. The tubes were gently swirled every 2 minutes during the incubation period. Two μL of the subcloned vector was added to the SURE® cells and allowed to incubate on ice for 30 minutes. The tubes were then heat pulsed in a 42°C water bath for 45 seconds to allow for maximal transformation efficiency to occur and subsequently chilled on ice for 2 minutes. A 0.9 mL aliquot of pre-heated SOC medium was added to the mixture and placed in a 37°C shaking incubator for 1 hour. The transformed cells were plated onto LB-ampicillin (100 μg/mL) agar plates with 0.5 mM IPTG and 40 μg/mL X-gal for colour screening and incubated overnight at 37°C. The plasmids harbouring vectors without inserts appeared blue while those with inserts appeared white.

4.6.2 Overnight Culturing of Bacterial Plasmids

Three white colonies from each plate were picked out and added to individual 15 mL Falcon tubes containing 5 mL of Luria-Bertani (LB) media supplemented with 150 μg/mL ampicillin. The bacterial plasmids were allowed to propagate overnight at 37°C in a shaking incubator. A sample of the bacterial culture was removed and stored in a 30% glycerol solution at -80°C.

4.6.3 Isolation of DNA Plasmids from Culture

The DNA plasmids were isolated using a QIAprep Spin Miniprep Kit (Qiagen, Mississauga, ON, Canada). After removing the cultures from an overnight 37°C shaking incubator, the cells were harvested by centrifugation at 3,200 x *g* for 15 minutes. The cells were resuspended in Buffer P1 and transferred into 1.5 mL microcentrifuge tubes. Lysis buffer P2 was added to release the DNA in which time the solution became viscous and clear. It was then deactivated by the addition of neutralization buffer N3 which made the solution cloudy. After centrifugation at full speed (12,000 x *g*) for 10 minutes to pellet the cellular debris, the supernatant was transferred to a QIAprep spin column. The column underwent centrifugation at 12,000 x *g* for 1 minute to allow the DNA to bind to the column membrane and then washed by Buffers PB and PE to remove nuclease activity and any contaminants and inhibitors. The DNA was eluted with 50 uL RNase-free, DNase-free water and stored at -80°C. DNA concentration was determined by diluting 10 µL of sample into 490 µL of sterile water and measuring the absorbance at 260 and 280 nm in triplicate on a UV spectrophotometer (Lambda 2 Spectrometer, PerkinElmer, Boston, MA, USA). The concentration and yield was calculated using Equations 1 and 2.

Equation 1.

$$[\text{DNA } \mu g/\mu L] = [(A260)(\text{dilution factor})(50\ \mu g/mL)]/1000$$

Equation 2.

$$\text{Amount of DNA} = ([\text{DNA}])(\text{vol. of sample})$$

4.6.4 Sequencing and Re-culturing of Bacterial Plasmid

A sample of the plasmid was submitted to the University of British Columbia's Nucleic Acid Protein Service (NAPS) Unit for sequencing. Once the correct sequence was confirmed, 10 µL of SURE® cells carrying viral plasmid were placed in a 50 mL Falcon tubes containing 15 mL of Luria-Bertani (LB) media supplemented with 150 µg/mL ampicillin to allow propagation of the bacteria which occurred overnight at 37°C in a shaking incubator. The DNA plasmids were isolated using the technique described in Section 4.5.3 with the following changes. After the cells were resuspended in Buffer P1, they were split into 3-1.5 mL microcentrifuge tubes. The DNA that was eluted was pooled, aliquoted and stored at -80°C.

4.6.5 Linearization of DNA Plasmids by Restriction Enzymes

Twenty µg of plasmid DNA was linearized using 40 U of Xba I for two hours at 37°C then heated at 65°C for 15 minutes to inactivate the Xba I. One µL each of uncut and linearized plasmid was run on an ethidium bromide enhanced 1% agarose gel to ensure complete digestion, as any residual uncut plasmid greatly decreases the yield of *in vitro* transcribed RNA.

4.6.6 *In vitro* Transcription of Digested DNA Plasmids

Two µg of digested plasmid was *in vitro* transcribed using a MEGAshortscript kit (Ambion, Austin, TX, USA). Transcription buffer, dNTPs, plasmid and enzyme mix were combined to a final volume of 20 µL and incubated for 2 hours. RNase-free DNase I was added to the reaction and allowed to incubate at 37°C for an additional 15 minutes to remove template DNA. To recover the transcript RNA, RNase-free water and ammonium acetate precipitation solution were added to the mix to terminate the reaction. Two volumes of 100% ethanol was added to

precipitate the RNA. The reaction mix was chilled at -20°C and underwent centrifugation for 15 minutes at 12,000 x *g*. The supernatant was carefully removed and the remaining alcohol allowed to evaporate. The RNA pellet was then resuspended in 100 µL of RNase- and DNase-free water. The RNA concentration was determined by diluting 10 µL of sample into 490 µL of sterile water and measuring the absorbance at 260 and 280 nm in triplicate on a UV spectrophotometer (Lambda 2 Spectrometer, PerkinElmer, Boston, MA, USA). The concentration and yield was calculated using Equations 3 and 4.

Equation 3.

$$[\text{RNA } \mu g/\mu L] = [(A260)(\text{dilution factor})(40\ \mu g/mL)]/1000$$

Equation 4.

$$\text{Amount of RNA} = ([\text{RNA}])(\text{vol. of sample})$$

The yield of these *in vitro* transcribed RNA transcripts were on the order of 10^{14} copies/µL. A working concentration of 10^7 copies/µL for each of the transcripts was prepared while the remaining amount of concentrated transcripts were aliquoted and stored at -80°C.

4.7 Reverse Transcription

For synthesis of complementary DNA (cDNA), 10 µL RNA per 20 µL reaction was reverse transcribed using random primers and Superscript II Reverse Transcriptase (Invitrogen, Burlington, ON, Canada). One µL each of random primers (2 µg/µL) and dNTPs (10mM)

(Invitrogen) were added to the RNA, incubated at 65°C for 5 minutes, and immediately placed on ice. The mixture was pulse centrifuged to recover any condensation built up on the walls and cap of the PCR tube. A master mix of 4 µL 5X first strand buffer, 2 µL 0.1M dithiothreitol (DTT) and 1 µL RNaseOUT Recombinant Ribonuclease Inhibitor (40U) (Invitrogen, Burlington, ON, Canada) was added to the RNA mixture and incubated at 42°C for 2 minutes. The addition of 1 µL of Superscript II Reverse Transcriptase (200U) (Invitrogen, Burlington, ON, Canada) completed the reaction mix. This was incubated at room temperature for 15 minutes, placed in the Robocycler (Stratagene, La Jolla, CA) and heated to 42°C for 50 minutes and 15 minutes at 70°C to complete the RT reaction. The cDNA produced from the *in vitro* transcribed transcripts were serially diluted from 10^8 to 10^1 copy numbers, aliquoted and stored along with the specimen cDNA at -80°C until use.

4.8 Quantitative Real-Time PCR on the ABI 7000

Originally, the qPCR assays were developed for the ABI 7900HT (Applied Biosystems, Foster City, CA, USA) machine. However, the machine was upgraded during assay development to an ABI 7900HT Fast system for use in the virology laboratory at Women's and Children's Hospital of British Columbia. It was then decided that a switch would be made to the older ABI 7000 instrument to complete the remaining experiments.

The ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was the platform used for quantitative qPCR. It utilized a tungsten-halogen lamp in a 96-well format. Target amplicon detection was via a plate reader that scanned the plate during each cycle and recorded the amount of fluorescence in each well.

The 25 μL reactions consisted of 2X TaqMan Universal PCR Master Mix (containing AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP, ROX passive reference and optimized buffer components), water, primer mix and probe and 5 μL cDNA template. Each viral assay had it own optimized concentration of primers and probe ranging from 300-600 nM and 100-200 nM, respectively. HPRT was used as an internal control to normalize the differences in the quantity of total cDNA in each sample. The negative control and standards with copy numbers from 10^7 to 10^1 were assayed in triplicate while the samples were analyzed in duplicate. The results were analyzed using the accompanying software, ABI Prism 7000 SDS (Applied Biosystems, Foster City, CA, USA).

Standardized cycling conditions were used that included an initial incubation of 2 minutes at 50°C, which allowed for AmpErase uracil N-glycosylase (UNG) to activate and decontaminate the reaction by cleaving uracil bases from DNA strands that have been synthesized in the presence of dUTP. This was followed by 10 minutes at 95°C to activate the DNA AmpliTaq Gold polymerase. Finally, 50 amplification cycles were carried out, each comprising of 15 seconds at 95°C for denaturation and 1 minute at 60°C for primer annealing and extension. The amount of fluorescence in each well was read by the machine during the annealing/extension phase. A standard curve generated from the serially diluted synthetic control allowed for the quantification of the unknown specimens.

Amplification early on in the assay between background and amplicon detection signals cannot be differentiated. Only after the amplicon has been replicated enough to enter its exponential phase, and crosses the threshold cycle can the assay's progress be examined. The threshold

cycle, Ct, is the point at which fluorescence reaches a detectable level and may be set anywhere in the log-linear phase of the reaction. Those samples with a Ct value of 50 did not have the amplification curve cross the threshold line and were considered negative. Samples with a value of less than 50 and were within the dynamic range of detection for a particular viral assay were considered positive as the amplification curve crossed the threshold line at that cycle. The starting concentration for each amplified virus in an unknown specimen was obtained from the corresponding Ct on the standard curve.

The efficiency of the reaction and dynamic range of detection can also be calculated. Theoretically, a well designed assay should have a slope between -3.1 and -3.6 (219). This corresponds to an efficiency of between 90 to 110%. The efficiency of a PCR reaction can be calculated by using equation 5:

Equation 5.

$$\text{Efficiency} = 10^{(-1/\text{slope})} - 1$$

There are numerous variables that affect PCR efficiency. This includes amplicon length, secondary structure, and choice of primers (220). The dynamic range of detection is then based on a log10-fold dilution series of virus. The more dilutions the qPCR assay can detect, the larger its dynamic range.

4.9 Data Analysis

Statistical analysis was performed using SPSS v.11.0 software (SPSS Corporation, Chicago, IL). Patients whose respiratory specimens yielded a negative result from the HPRT assay were excluded from further testing and analysis because it would not be possible to determine whether a negative viral qPCR result was due to true absence of virus or due to an inadequate specimen. Viral load data underwent a logarithmic transformation to better approximate a normal distribution. The statistical significance of the differences between the logarithmically transformed mean qPCR viral loads between the culture negative and culture positive groups was determined using a Student's t-test. The significance of the differences between viral load and clinical diagnosis (URTI, fever, LRTI) was determined using an analysis of variance (ANOVA), followed by pair-wise Student's t-tests. In all analyses, a p-value < 0.05 was considered to be statistically significant.

Table 2. Summary of viral assay primers, probe and amplicon length.

Virus/Host Gene	Accession #	Forward Primer (5' – 3')	Reverse Primer (5' – 3')	Probe (5' – 3')	Gene Segment	Amplicon Length
Influenza A	AY210270	CAT GGA RTG GCT AAA GAC AAG ACC	AGG GCA TTT TGG ACA AAK CGT CTA	TGC AGT CCT CGC TCA CTG GGC ACG	Matrix (M2) gene	126
Influenza B	X00897	AAA TAC GGT GGA TTA AAT AAA AGC AA	CCA GCA ATA GCT CCG AAG AAA	CAC CCA TAT TGG GCA ATT TCC TAT GGC	HA gene	171
PIV-1	AF117818	GAT CCA GCA GTC GCA GCT CTA	ATG CCC AAT CTA GGA AGC TTG A	AGA TTC ATC AAG GCA GGT CTG TTA GAT AAG CAG GT	Large (L) gene	124
PIV-2	X57559	TCA CCC CTG AAC TTG TTA TTT GTT T	GCA GAG AGC GGT GAC ATT CAT	TAA TGG TAA GTG ACA TGT TTG AG	Large (L) gene	122
PIV-3	Z11575	GCT GGA ATG TTA GAT ACG ACA AAA TC	TCG CAA AGT CCT ACT TAG TGT TTC A	TAA TTC GGG TTG GCA TAA	Large (L) gene	132
RSV A	M11486	GCT CTT AGC AAA GTC AAG TTG AAT GA	GCC ACA TAA CTT ATT GAT GTG TTT CTG	ACA CTC AAC AAA GAT CAA CTT CTG TCA TCC AGC	N gene	144
RSV B	D00736	GAT GGC TCT TAG CAA AGT CAA GTT AA	TGT CAA TAT TAT CTC CTG TAC TAC GTT GAA	TGA TAC ATT AAA TAA GGA TCA GCT GCT GTC ATC CA	N gene	104
MPV	AF371337	ACC GTG TAC TAA GTG ATG CAC TCA A	CAT TGT TTG ACC GGC CCC ATA A	CTT TGC CAT ACT CAA TGA ACA AAC	N gene	213
HCoV-229E	X15498	TTT ATG GTT TGA AGA TGC TTG TAC TGT	TAC GGC CAT AAA AAA GCT AAA TGC	AAT CTT TGA CAC CTG GGC TA	Membrane (M) gene	128
HCoV-OC43	M93390	GGA CTA TCA TAC TCT GAC GGT CAC AA	GGT GTG TAA CCT TAG CAA CAG TCA TAT AA	ACT AGG TAC TGG CTA TTC TTG GGC AGA TTT GC	Membrane (M) gene	125
HRV	AY751783	GTG AAG AGC CSC RTG TGC T	GCT SCA GGG TTA AGG TTA GCC	TGA GTC CTC CGG CCC CTG AAT G	5' untranslated region	68
Adenovirus	L19443	GCC CCA GTG GTC TTA CAT GCA CAT C	GCC ACG GTG GGG TTT CTA AAC TT	TGC ACC AGA CCC GGG CTC AGG TAC TCC GA	Hexon gene	132
HPRT	NM000194	GAA AGG GTG TTT ATT CCT CAT GGA	CTT GAG CAC ACA GAG GGC TAC A	ATC TCG AGC AAG ACG TTC AGT CCT GTC CAT A	Exon 2-3	111

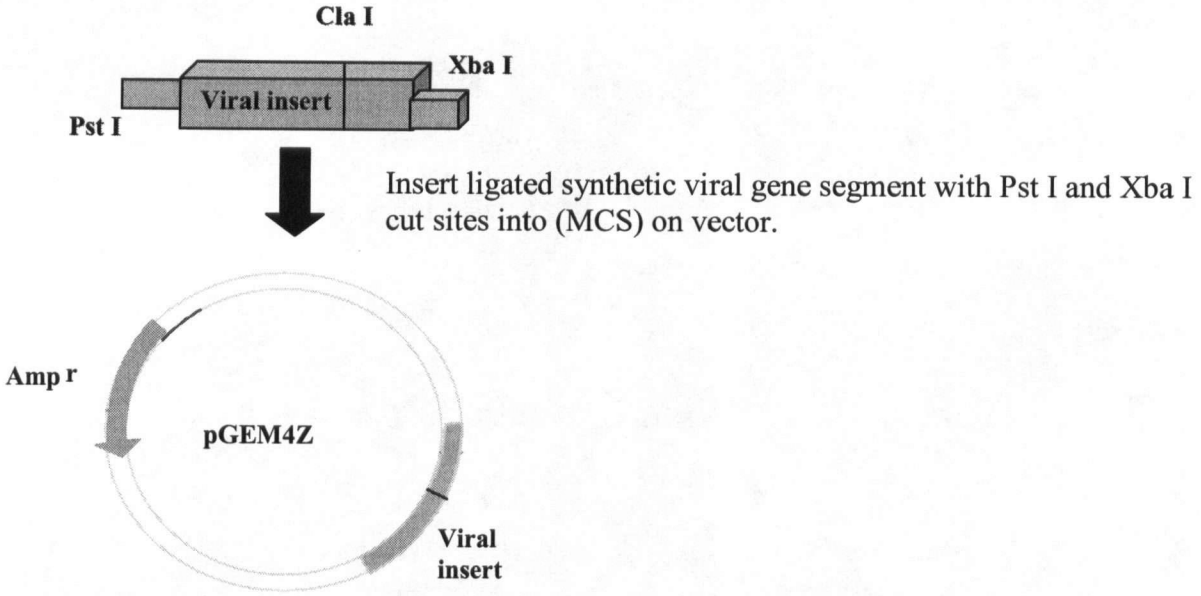

Figure 1. A schematic cartoon of synthetic control preparation. cDNA for each respiratory virus within the panel was digested using Pst I and XbaI restriction enzymes on the 5' and 3' ends respectively. The viral insert was then cloned into a pGEM4Z vector that had also been digested using Pst I and Xba I.

5. RESULTS

5.1 Efficiency and Dynamic Range of Detection of Viral Assays

The efficiency was first assessed on the ABI 7900HT using RNA isolated from reference strain of viruses. Also, the efficiency and dynamic range of detection for each assay was assessed using the control plasmids on the ABI Prism 7000. **Table 3** compares the average efficiency and R-squared value of each assay between the ABI 7900HT and ABI Prism 7000. Overall efficiencies and reproducibility on both instruments were high and therefore the decision was made to keep the assays as developed. **Table 4** illustrates the corresponding range of detection based on use of the ABI 7000 instrument. While it is possible to redesign and reconfigure the assays to eventually conform to guidelines for efficiency (219), each of the assays could detect its target virus sequence to a low viral copy number (10^1 or 10^2 copy numbers in a specimen) as shown in **Table 4**. **Figure 2** illustrates a typical amplification curve; this particular curve represents the synthetic control of influenza A with a dynamic detection range of $1.96x10^2$-$1.96x10^7$.

5.2 Cross-reactivity of qPCR Primers and Probes

Each viral assay was validated for cross-reactivity by testing each specific assay against all the developed synthetic control plasmids. Prior to machine validation, every primer and probe sequence was 'blasted' through NCBI for specificity to its particular virus to ensure no cross-reactivity with published sequences to other viruses or the human genome.

Initial cross-reactivity tests were performed on the ABI 7900HT and subsequently on the ABI Prism 7000. Results came back identical between machines and showed no cross-amplification between any of the viral assays, with the only exception being HRV. HRV belongs to the

picornaviridae family, to which enteroviruses are also categorized. It is very common to find PCR cross-amplification between the genera (221). When a reference strain of an enterovirus, coxsackievirus B3, was used the HRV assay showed minor amplification, with a Ct of approximately 38. Importantly, this was very easily distinguishable from true HRV test positives as the dynamic range of detection ended at a Ct of approximately 33.

5.3 Assessment of Amplifiable Material by Host Gene HPRT

Results of the reference gene assay showed that out of 100 culture negative specimens initially chosen, five specimens were actually culture positive for HRV and one specimen was negative for HPRT. These were replaced with six other test specimens to keep the total number of evaluated culture negative specimens at 100. For the culture positive specimens examined, one influenza B culture isolated specimen returned a negative result on the HPRT assay and was subsequently excluded from further examination as insufficient amplifiable material was present. Out of the total 365 clinical specimens tested, only those two specimens had negative outcomes for HPRT such that the overall HPRT detection rate was 99.5%.

The mean HPRT Ct was 34.15 with a 95% confidence interval of 33.70-34.60. Given this very narrow range, qPCR viral loads were subsequently interpreted per starting volume of nasal specimen, analogous to how HIV viral loads are interpreted as HIV copy number per mL blood. **Figure 3** illustrates a typical amplification plot for HPRT.

5.4 Relationship of qPCR Results to Positive Viral Culture Results

Sensitivity is defined as the proportion of people with disease who return a positive test result. A sensitivity of 100% would be interpreted as all patients with a disease who are diagnosed as such, without presence of false negatives (i.e., patients with disease but for whom test results are negative). It is calculated using the following formula (222):

Sensitivity = number of true positives / (number of true positives + number of false negatives)

Table 5 shows sensitivity results for each of the viral assays in which "true positives" are defined by viral culture results. All the assays demonstrated a high degree of sensitivity with the exception of PIV-3 which had sensitivity of 80%. In addition, one of the PIV-3 culture positive specimens that was negative by qPCR instead came up positive at a high viral load for HCoV-OC43. With respect to RSV, the viral culture method did not distinguish between subtypes A and B. Of the 40 RSV culture positive specimens studied, 39 were positive for at least one subtype of RSV by qPCR, and one specimen positive for both.

Specificity is defined as the proportion of people without disease who return a negative test result. A specificity of 100% is interpreted as healthy or undiseased patients being diagnosed as such. It is calculated using the following formula:

Specificity = number of true negatives / (number of true negatives + number of false positives)

However, since healthy individuals do not typically come to the hospital and provide respiratory specimens, the study design did not allow for determination of specificity of the qPCR assays. Furthermore, in this particular laboratory system, the concept of specificity is problematic because viral culture is an imperfect gold standard. For example, a negative culture result cannot be interpreted as the patient having no disease since many viral pathogens do not grow well in culture or can undergo inactivation *ex vivo*.

5.5 qPCR on Culture Negative Specimens

Of the 100 culture negative specimens examined, qPCR amplified at least one virus in 43 (43%) of the specimens. Further analysis of these 43 specimens showed that 36 specimens amplified one virus, 5 amplified two viruses and 2 specimens amplified three viruses. The specific viruses that were isolated from these specimens are shown in **Table 6.** HRV was the most commonly detected virus in the screen with 20 specimens amplifying viral nucleic acid followed by HCoV-OC43 with 13 specimens positive. MPV was found in 7 specimens tested. Less commonly identified viruses were adenovirus and PIV-3 at 3 specimens each, RSV B with 2 and RSV A, influenza A, influenza B and HCoV-229E each with a lone positive identification.

5.6 Virus Culture Negative versus Virus Culture Positive Specimens

Table 7 shows the relationship between viral load and culture negativity or positivity in the human respiratory specimens studied. Not surprisingly, culture-positive specimens for the most part had statistically significant higher viral loads than culture-negative specimens. Influenza A, PIV-3, RSV A, RSV B, HRV and adenovirus all had p-values < 0.05. In addition, viral copy numbers below approximately 10^4 copies/mL in nasal specimens tended to be associated with

negative culture results, with the notable exception of HCoV-OC43, in which cultures were uniformly negative despite a mean viral load of $10^{6.2}$ copies/mL. Also, PIV-1 had a mean viral load of $10^{3.52}$ copies/mL; the only virus that had a positive culture mean viral load below 10^4copies/mL.

5.7 Relationship of Viral Load and Clinical Diagnosis

Table 8 outlines the relationship between viral load and clinical diagnosis, URTI, fever, or LRTI. The 95% confidence intervals of qPCR viral loads did not overlap between the three groups. The p-value between URTI and LRTI was <0.001, between URTI and fever <0.009 and between fever and LRTI <0.004. An ANOVA revealed that all three groups were statistically different from each other with a p-value < 0.001.

Table 3. Efficiencies and R-squared values of qPCR protocols as performed on two different instruments.

	ABI 7900HT		ABI Prism 7000	
Virus	**Efficiency (%)**	**R^2**	**Efficiency (%)**	**R^2**
Influenza A	93.8	0.995	93.3	0.995
Influenza B	107.2	0.990	95.1	0.985
PIV-1	94.5	0.989	93.9	0.993
PIV-2	96.5	0.994	96.5	0.973
PIV-3	104.4	0.994	77.4	0.966
RSV A	98.0	0.958	76.8	0.990
RSV B	102.0	0.996	73.7	0.989
MPV	98.8	0.967	82.0	0.997
HCoV-229E	89.9	0.979	82.3	0.993
HCoV-OC43	89.6	0.950	85.4	0.922
HRV	ND	ND	150.7	0.999
Adenovirus	92.3	0.956	76.5	0.996

ND: not done, as the HRV assay was developed after the switch to the ABI 7000 instrument.

Table 4. Range of detection of viral assays on the ABI Prism 7000.

Virus	Range of Detection (viral copy numbers)
Influenza A	$1.96x10^{2}$-$1.96x10^{7}$
Influenza B	$1.69x10^{2}$-$1.69x10^{7}$
PIV-1	$2.34x10^{1}$-$2.34x10^{7}$
PIV-2	$2.27x10^{1}$-$2.27x10^{7}$
PIV-3	$1.82x10^{2}$-$1.82x10^{7}$
RSV A	$2.23x10^{2}$-$2.23x10^{7}$
RSV B	$3.44x10^{2}$-$3.44x10^{7}$
MPV	$1.49x10^{2}$-$1.49x10^{7}$
HCoV-229E	$2.17x10^{2}$-$2.17x10^{7}$
HCoV-OC43	$2.55x10^{2}$-$2.55x10^{7}$
HRV	$2.32x10^{1}$-$2.32x10^{7}$
Adenovirus	$1.60x10^{2}$-$1.60x10^{7}$

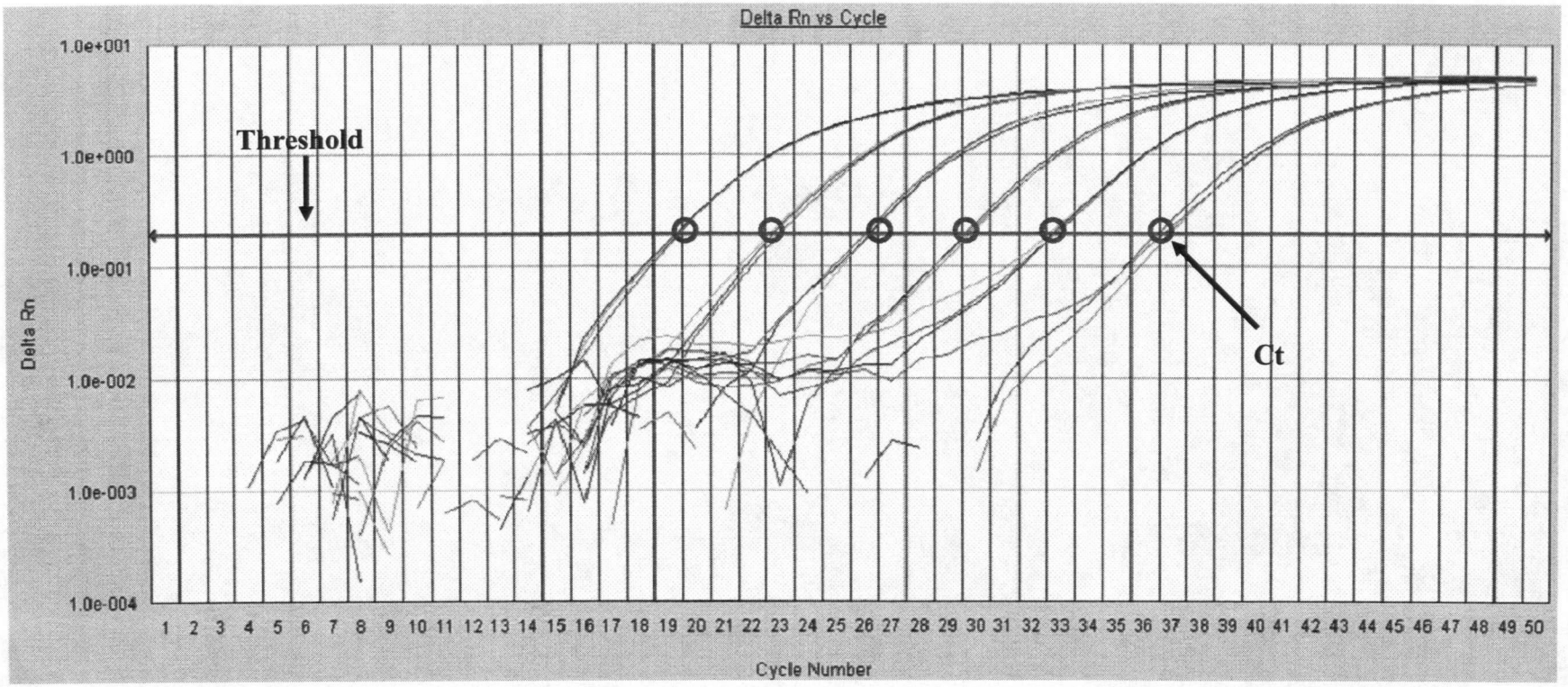

Figure 2. Representative real-time qPCR viral amplification curve for Influenza A. A ten-fold dilution series (10^7-10^2) of Influenza A synthetic control was amplified in triplicate. The horizontal green line represents the default set threshold. The point at which the amplification curve crosses the threshold is called the threshold cycle (Ct) and is the point at which fluorescence is detectable above background levels. Delta Rn is the magnitude of the signal generated during the qPCR.

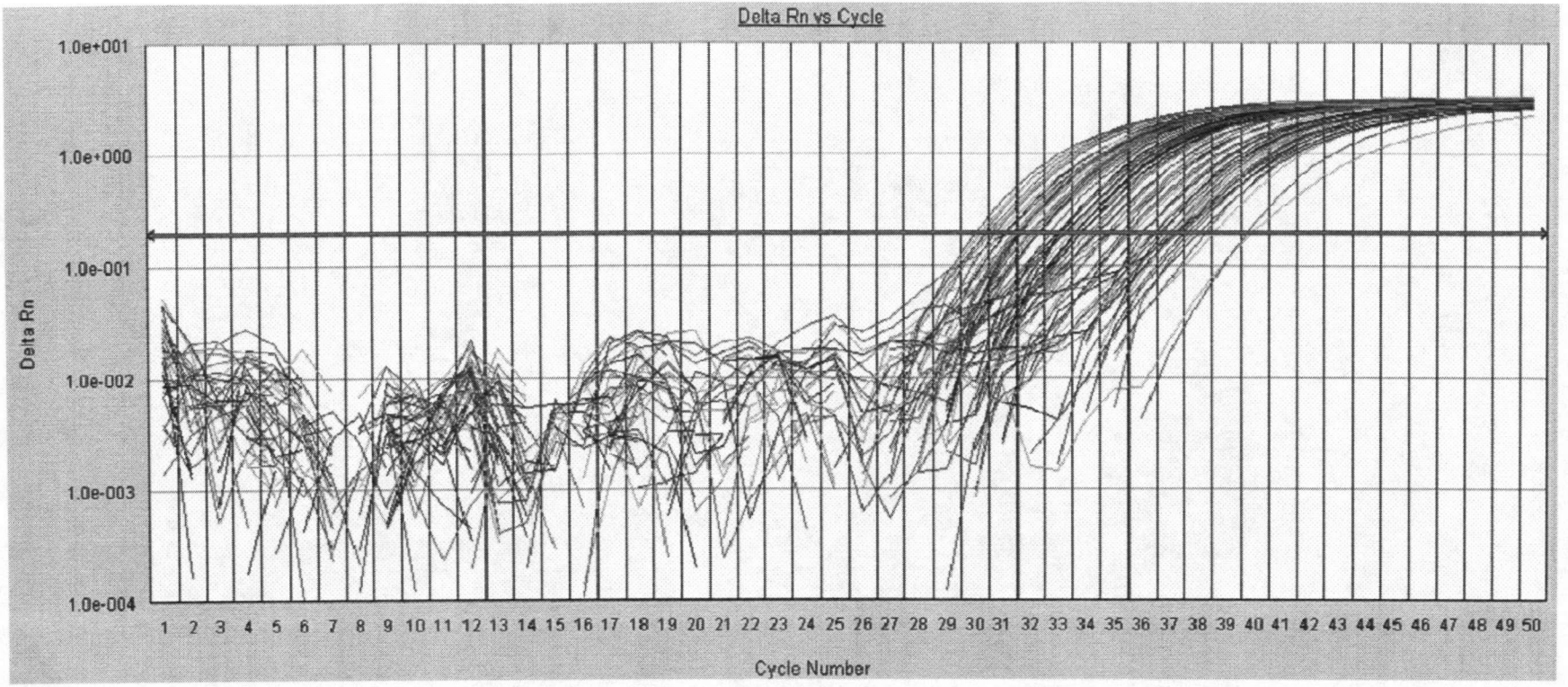

Figure 3. HPRT amplification curve. Real-time qPCR amplification plot of HPRT from cDNA produced from the isolated RNA of archival NPA specimens. Each specimen was run in duplicate.

Table 5. Sensitivity of qPCR viral assays.

Virus	n	True Positives (viral culture)	False Negatives	Sensitivity (%)
Influenza A	30	29	1	96.7
Influenza B	29	28	1	96.6
PIV-1	40	36	4	90
PIV-2	40	40	0	100
PIV-3	40	32	8	80
RSV *	40	39	1	97.5
MPV	0	-	-	n/a
HCoV-229E	0	-	-	n/a
HCoV-OC43	0	-	-	n/a
HRV	5	5	0	100
Adenovirus	40	40	0	100

*: consisted of RSV A (n = 21), RSV B (n = 17) and RSV A + RSV B (n = 1) by qPCR

n/a: not applicable

Table 6. Summary of the types of respiratory viruses identified using qPCR screen in the culture negative specimens.

Virus	Number of Culture Negative Specimens that were Positive by qPCR
Influenza A	1
Influenza B	1
PIV-1	0
PIV-2	0
PIV-3	3
RSV A	1
RSV B	2
MPV	7
HCoV-229E	1
HCoV-OC43	13
HRV	20
Adenovirus	3

Table 7. Logarithmically transformed qPCR loads and their relation to previously confirmed culture negative and culture positive specimens.

Virus		n	Log mean qPCR load (95% CI)	p-value
Influenza A	culture (-)	1	4.06	0.004
	culture (+)	29	6.38 (6.10-6.66)	
Influenza B	culture (-)	1	3.06	0.25
	culture (+)	28	4.54 (4.05-5.03)	
PIV-1	culture (-)	0	-	n/a
	culture (+)	36	3.52 (3.22-3.82)	
PIV-2	culture (-)	0	-	n/a
	culture (+)	40	6.09 (5.52-6.66)	
PIV-3	culture (-)	3	2.73 (2.57-2.90)	<0.001
	culture (+)	32	7.65 (7.18-8.12)	
RSV A	culture (-)	1	3.78	0.002
	culture (+)	22	7.49 (7.02-7.96)	
RSV B	culture (-)	2	3.28 (2.40-4.15)	<0.001
	culture (+)	18	7.63 (7.21-8.05)	
MPV	culture (-)	7	4.20 (3.30-5.11)	n/a
	culture (+)	0	-	
HCoV-229E	culture (-)	1	3.09	n/a
	culture (+)	0	-	
HCoV-OC43	culture (-)	13	6.20 (4.94-7.47)	n/a
	culture (+)	0	-	
HRV	culture (-)	19	2.43 (1.30-3.56)	0.032
	culture (+)	5	5.21 (1.86-8.55)	
Adenovirus	culture (-)	3	3.38 (0.61-6.14)	0.002
	culture (+)	40	7.42 (6.76-8.09)	

Table 8. Logarithmically transformed qPCR loads and their relation to clinical diagnosis.

Diagnosis	n	Log mean qPCR load (95% CI)
URTI	23	2.49 (1.03-3.94)
Fever	98	4.62 (4.03-5.22)
LRTI	66	5.90 (5.28-6.53)

p-values:

URTI vs. fever: <0.009
URTI vs. LRTI: <0.001
fever vs. LRTI: <0.004

6. DISCUSSION

This thesis looked to accomplish a number of aims. The development of a quantitative qPCR respiratory panel along with the selection of a suitable reference gene that used standardized conditions was successfully achieved. Synthetic controls for each respiratory virus of interest were created and provided accurate quantification of viral loads. Using archival respiratory specimens, we were able to determine the sensitivity of our assays as compared to viral culture and determine viral load ranges associated with positive or negative culture results. Lastly, we tried to ascertain whether or not there was a significant relationship between a patient's clinical diagnosis and viral loads detected by qPCR. As noted in the results section, some interesting findings were uncovered and these will be discussed further.

With respect to the ability to use the qPCR protocols on different instruments, there was, in general, good performance in terms of efficiency and reproducibility on both the ABI 7900HT and ABI Prism 7000. The variations may be attributed to reasons unrelated to instrumentation such as variance in pipetting technique or the type of template used. The initial efficiency assessment on the ABI 7900HT was conducted using RNA isolated from reference strains of virus which contained not only viral RNA but also cell genomic DNA and its RNA, which is more representative of the test specimens collected from patients. Synthetic controls were not used in the initial efficiency tests on the ABI 7900HT since the primer and probe set for each viral assay needed to be validated first. Only after the viral assay was shown to be in working order, were the corresponding synthetic plasmids produced. The efficiency tests on the ABI 7000 were performed using synthetic templates which contained only the pure target and are therefore

more accurate for quantification. Overall, the results demonstrate the ability to readily transfer qPCR assays onto a different instrument with good outcomes.

In general, there was good linearity over a minimum of six log units for each assay. Theoretically, efficiency should be between 90 to 110% while the R^2 value should be above 0.99; however valid data may be obtained from values outside this range. Influenza A and PIV-1 were the only 2 assays to stay within the theoretical criteria on the 7000 instrument. The reproducibility of influenza B, RSV A, RSV B, MPV, HCoV-229E, HRV and adenovirus assays were all greater than 0.99 which was taken as the deciding factor to keep the assays as they were. The PIV-2, PIV-3, and HCoV-OC43 assays may be considered for re-design in future prospective studies. This may be due to differences in instrumentation detection as these assays would have been acceptable based on results on the ABI 7900HT. Despite these limitations, all of the assays consistently had a wide range of detection and were reproducible.

Each assay was assessed against all synthetic controls made for each of the viruses in the qPCR panel. No cross-reactivity between the viral assays was found amongst the synthetic controls as highly conserved regions of the viral genome were utilized in preparation of the plasmids. As mentioned in section 5.2, HRV had some minor cross-amplification with a reference strain of CVB3, an enterovirus which is in the same family, picornaviridae. As only five of over 100 HRV serotypes have been sequenced, it is difficult to design an assay that will detect as many rhinoviral serotypes as possible without amplifying the occasional enterovirus. A BLAST search of the HRV primers and probe revealed that the CVB3 sequence could be detected by the probe but the E (Expect) value was greater than that of numerous HRV serotypes. The E value

describes the number of hits that may be expected by chance when searching a database of a particular size. The closer the E value is to zero, the more significant the match is. HRV serotypes have an E = 1e-04 while CVB has an E = 5e-04.

This study clearly demonstrated that amplification of HPRT for NPA specimens was consistent and showed little variation. This finding is in contrast to results of other studies (223-225) in which the reference genes chosen have been found to be regulated by the experimental conditions or are so highly expressed that normalization is incorrect and unsuitable. In addition, the Ct values of HPRT were expressed within the range of detection of the NPA test specimens and therefore allowed for detection of any minute changes in gene expression. This study also established the ability to interpret qPCR viral loads per starting volume of nasal specimen. Further studies are required to determine if use of HPRT as a reference gene can be extended to other types of respiratory specimens including human lung tissue.

The sensitivity of all the assays compared to culture was high with values greater than 96% with the exception of the PIV-1 and PIV-3 assays. This could be due to the viral load of the false negative specimens is between the threshold levels of culturing negative or positive isolates. While this was an unknown for PIV-1 as none of the 100 culture negative specimens gave a positive result; the mean log qPCR load between the negative and positive cultures for PIV-3 were 2.73 and 7.65 respectively. The large disparity in mean viral loads was an interesting result as perhaps only a swab taken at peak infection will return a positive result on culture. Development of another assay may provide further insights into this issue.

The effect of freeze-thaw cycles on viral RNA content in archival NPA was not investigated in this study. However, other studies have reported similar values in viral concentration between freshly processed specimens and those that have undergone freeze-thaw cycles after being stored for 16 months (226, 227). Viral RNA exhibited a decrease in viral load with an increase in the number of freeze-thaw cycles while viral cDNA that was freeze-thawed multiple times returned similar load values to that of freshly prepared cDNA except at lower copy numbers (i.e., 10^1-10^2) as viral titres tend to fall with repeated freeze-thawing. Repeated freezing and thawing of specimens is a technical consideration in use of qPCR for quantitative viral load measurement.

Overall, in seven specimens, nucleic acid from more than one respiratory virus was amplified. As these test specimens were only taken at one particular time point, it was not possible to determine at which time the patient was exposed to a specific virus.

It is interesting to note in that out of the 43 culture negative specimens in which viral nucleic acid was amplified by qPCR, 11 were positive for viruses that routinely undergo tests by culture and serology. This indicates that potentially a significant number of viral infections go undetected by "gold standard" methodology. However, literature has shown that viral culture may not necessarily be suitable as a "gold standard" for certain respiratory viruses. Johnston and colleagues found that a number of HRV detected by PCR went undetected using culture because some strains were very slow growing (228). Because HCoVs tend to grow slowly on specialized cells and are not well adapted to conventional cell culture methods, they do not undergo routine culturing in the majority of diagnostic or virology laboratories (229). This has potential implications interpreting the epidemiology of other studies of respiratory viruses. A greater

percentage of illness and disease may in fact be attributable to viral infections and therefore is currently under-reported. The New Vaccine Surveillance Network, as reported by Griffin, Iwane and colleagues, found that using both viral culture and RT-PCR, 61% of children who are hospitalized have a respiratory viral infection leaving 39% unassociated with a detectable pathogen (214, 230). Concerning MPV, the percent of MPV positive specimens (7%) found in the culture negative clinical specimens mirror the percentages found in some published epidemiological studies conducted thus far (39, 93, 96). However the assay modified from Mackay et al. (217) is not based on the reference strain of MPV found in the National Center for Biotechnology Information (NCBI) database A current study utilizing an assay based on the reference strain of MPV showed a detection rate of 16.2% (231) while Mackay et al. found MPV in 9.7% of specimens tested. Therefore, this study may underestimate the number of MPV positive specimens.

For the 57 culture negative specimens in which no viral nucleic acid was detected by qPCR, infection might have involved a pathogen not tested by the qPCR panel, or the test specimen had a viral load that was below the sensitivity of detection by the technique. The presence of PCR inhibitors may also contribute to a false negative result and this may be tested by adding a known amount of virus to an aliquot of the culture negative specimen to see if it amplifies as expected.

For the first time, a correlation between viral load and concomitant positive culture results for several viruses has been presented in this thesis. A range of viral loads was confirmed for 6 viruses that included influenza A, PIV-3, RSV A, RSV B, HRV and adenovirus. Based on the ranges established for each virus, a viral load with a value below the determined threshold is

likely to return a negative culture result. This result builds upon the results of the culture negative specimens discussed earlier, in which a number of viral infections go undiagnosed using traditional methods of detection. Further studies are necessary to definitively address this issue.

The results of this thesis also show for the first time a relationship between viral load as determined by the qPCR panel and a patient's clinical diagnosis as determined by requisition forms. The results suggest that patients with a more severe disease have higher viral loads but further large-scale studies are required to confirm this possibility.

There are certain potential problems that may arise using NPAs to determine viral load. In blood and serum, nucleic acid extraction tends to be straight-forward as the specimen is relatively homogeneous. NPAs and other body fluids tend to be heterogeneous with potentially large variability in specimen composition. A cytomegalovirus (CMV) study found that positive BAL viral culture results could not determine between viral shedding without disease and CMV pneumonitis (232). This study also found that viral loads determined in blood had some clinical value versus BAL which needs further study. In addition, blood and serum may be easily stabilized prior to transport and processing while NPAs are not. This may potentially lead to purified nucleic acid that is degraded or subject to degradation or may co-purify inhibitors of reverse transcription or PCR. Sample quality plays a large part in generating valid data as suboptimal nucleic acid increases the possibility of false-negative results. However, our results with using HPRT as a reference value, indicated that these potential issues were of little concern in this particular thesis.

The results of this study also suggest that qPCR is a robust, multi-faceted and sensitive technique. Once it is carefully standardized and validated, qPCR may in the future be used in conjunction with or as a replacement for other laboratory methods that are not as sensitive or unable to identify certain pathogens as other studies have reported (233, 234), particularly as virus-specific treatments are developed. Current detection methods based on culture are sometimes of limited clinical use as the time necessary to generate results takes far longer than the duration of illness itself. We have developed a respiratory viral screen that not only quantifies viral copy numbers but has resulted in new information concerning the correlation of viral load to culture results and viral load to clinical diagnosis. While our studies looked at a subset of specimens based only on diagnoses from requisition forms, it would be interesting to consider any associations between infections based on chart reviews or discharge diagnoses. Additionally, further studies conducted in a prospective manner, rather than retrospective, are required to validate the current findings.

The reported prevalence of respiratory virus infection varies in the literature. This can be attributed to a number of factors such as: type of test specimen used for diagnostic testing, type of diagnostic test used, patient population studied, and time of year. A recent study compared NPA, nasopharyngeal swabs (NPS), and oropharyngeal swabs (OPS) and the ability of these specimens to detect respiratory pathogens (235). The findings illustrated that NPA were better than NPS and were superior to OPS. A comparison between real-time RT-PCR versus culture results demonstrated that real-time RT-PCR detected a respiratory pathogen in 63% of the RTI episodes versus 21% by culture (236). For LRTI, these numbers differed even more with real-time RT-PCR detecting a respiratory pathogen in 73% of the specimens versus 9% by culture. A

look at the distribution of respiratory viral infections by age showed that there was a higher number of infections in patients who were under the age of 1 compared to those over the age of 21 (237). In terms of patients who were immunocompromised, the opposite result was found as the percent of respiratory infections increased from 12.5 in those 2-5 years of age to 25.6 in those 6-20 years of age. This particular study also revealed that HCoV-229E was detected solely in one season while HCoV-NL63 was detected only in another, with HCoV-OC43 distributed in both. Another study following a cohort of children in determining picornaviral infection with illness and effect of season found infection rates highest in the fall with similar rates found between the winter, spring and summer seasons (238). Clearly, interpretation of results in literature requires careful appraisal to elucidate the true prevalence of respiratory infection.

Some issues concerning the interpretations of these results arise with the lack of a healthy population of children for comparison. Future validation studies of the qPCR panel will need to include a group of children who are asymptomatic (healthy). A recent study by Falsey and colleagues matched symptomatic and asymptomatic patients in the diagnosis of RSV and MPV (239). They found a significantly higher rate of detection of respiratory infection in those with respiratory illness versus those without. Since healthy children are usually not brought to the emergency room, a study recruiting volunteers or a longitudinal study where children are brought in every week/month and followed for a number of years is needed – though a study of this magnitude would be particularly difficult to accomplish. Another possibility would be to have "matched" asymptomatic children in the population with those children who have NPA taken during symptomatic illness. A more comprehensive look at the epidemiology of respiratory

illness and disease may now be undertaken with the increase in sensitivity and quantification of qPCR.

Another point of interest is clinical relevance of real-time qPCR at low viral loads and the detection of multiple infections. For respiratory viruses like RSV and adenovirus, it is well known that these pathogens may persist in the lung and replicate at low levels long after the primary infection has subsided (145, 171). They are able to evade the host immune system and can act as a conduit for subsequent clinically symptomatic infection. Based on the viral loads, there is the ability to distinguish between current infection – those with an elevated viral titre, versus persistent infection – those that languish at a low level, through consecutive test specimens. A longitudinal study involving repeated sampling would help to discern any potential significance of multiple viruses documented by qPCR.

While real-time qPCR may have some advantages over conventional methods of viral diagnosis in terms of time and effort, cost is currently a large hindrance; therefore, qPCR may not yet be feasible for many laboratories. This is mainly due to the fact that most specimens are run in either duplicate or triplicate, standard curves are run in every plate, the development of probes does not always work, reagents, plastic consumables and the machine are costly. Applied Biosystems (Applied Biosystems, Foster City, CA, USA) is the industry leader in real-time PCR technology, with thermocyclers, reagents, plastic consumables, assay-by-design as some of their offered goods and services, and with this comes a high cost. However, alternative reagents, consumables, and platforms being offered by competing companies have helped drop the overall cost per specimen making the use of qPCR more affordable and attainable to smaller budget

laboratories. Experiments conducted within our laboratories have found comparable if not better results with these alternative reagents. With all this taken into consideration, qPCR, for the time being, is probably best left to dedicated virology laboratories for use in research, assay development, and validation.

Another diagnostic method which shows promise in further advancing multiple respiratory pathogen detection includes the Luminex® microsphere system (Luminex Corporation, Austin, TX). Up to 100 separate sets of colour-coded beads, "carboxylated microspheres", are each coated with a specific probe and the median fluorescent intensity (MFI) is measured using the Luminex compact analyzer. A low MFI (<500) is considered negative while a high MFI (>1000) is interpreted as a positive result. The advantages of the Luminex technology is the ability to multiplex a large number of reactions with a reduction of cost as less reagent, test specimen, time and labour are needed. Further studies are needed to validate this platform toward translation into the clinical diagnostic laboratory setting.

The simplicity in design of this respiratory qPCR panel allows for assays of new, relevant pathogens to be added and analyzed with ease. For example, a recent study in Hong Kong investigated the types of coronaviral infections found in patients (240). Out of 4,181 specimens tested, 87 were positive for HCoV, 53 positive for HCoV-OC43, 17 positive for HCoV-NL63, 13 positive for CoV-HKU1, and 4 positive for HCoV-229E. The occurrence of infection mirrors the results in this thesis as 13 specimens were positive for HCoV-OC43 and only 1 HCoV-229E specimen was positive in all the specimens examined. The number of HCoV-NL63 positive results is enough to warrant further examination and perhaps insertion into our current

respiratory qPCR screen as it is clearly found in greater numbers than HCoV-229E positives which are generally tested for when investigating coronaviral infections. Other advantages of this qPCR panel are that it can be adapted to a 384-well plate format, can be multiplexed, can be modified to a real-time qRT-PCR screen and can be transferred onto another detection platform if so desired.

In conclusion, we have developed a panel of real-time qPCR protocols for the detection and quantification of nucleic acids for 12 common human respiratory viruses. We have successfully established a qPCR respiratory viral panel using standardized conditions across detection platforms, and developed synthetic plasmids for each respiratory virus. Our results provide new information regarding the relationship of viral load to culture positivity and clinical symtomatology. Overall, the real-time qPCR panel described in this thesis contributes to the rapidly growing field of various molecular techniques involved in the diagnosis of viral infections.

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