THE CONTRIBUTION OF HUMAN METAPNEUMOVIRUS TO RESPIRATORY TRACT DISEASE

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To those closest to my heart

whose belief has remained

unfathomable

Contagion

Elephants are contagious!
Be careful how you tread.
An Elephant that's been trodden on
Should be confined to bed!

Leopards are contagious too.
Be careful tiny tots.
They don't give you a temperature
But lots and lots - of spots.

The Herring is a lucky fish
From all disease inured.
Should he be ill when caught at sea;
Immediately - he's cured**!**

Spike Milligan

Abstract

This thesis describes the development and implementation of an internally controlled one-step real-time RT-PCR assay for the diagnosis of human metapneumovirus (HMPV) and expansion of the clinical virology diagnostic service following the evaluation of newer multiplex methodologies, which revealed that traditional methods of virological diagnosis failed to diagnose approximately 50% of respiratory infections attributed to new and well-established viruses. The work presented here reiterates the contribution of HMPV to the burden of respiratory disease within the paediatric population, which accounted for 4.6% of acute respiratory infections previously not attributed to known respiratory pathogens in hospitalised children during the 3 year period investigated.

HMPV co-infection with other respiratory viruses is widely reported but few studies have considered the importance of bacterial co-infection in HMPV-associated respiratory infection. The frequency of bacterial co-infections with HMPV in children hospitalised with symptoms of acute respiratory infection was determined to provide evidence to support a potential role for commensal flora of the nasopharynx in co-infections with HMPV. HMPV was identified more commonly with one or more concomitant bacteria than as a sole respiratory pathogen or in combination with other viruses, which suggests the frequent involvement of HMPV in the development of bacterial co-infection. *Haemophilus influenzae* and *Streptococcus pneumoniae* were found frequently with HMPV but only *H. Influenzae* was significantly associated with HMPV. These findings may reflect complex changes in the epidemiology of *S. pneumoniae* since the introduction of the 7-valent pneumococcal conjugate vaccine into the routine childhood immunisation programme in England and Wales in 2006.

Phylogenetic analysis of sequences within the variable glycoprotein gene and conserved fusion gene were conducted to determine the genetic variability of HMPV lineages circulating within the paediatric cohort within the locality of Norwich and revealed the frequent displacement of the predominant circulating sublineage. Additionally, a unique strain circulated within the cohort, which suggests local factors influence HMPV circulation. Other strains identified were similar to strains circulating globally but predominant sublineages circulating within the cohort reflect those dominating within the UK and Ireland suggesting parallel changes in the circulation pattern of sublineages within the same geographical region.

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A	Adenine
aa	Amino acid
ABI	Applied Biosystems
AIDS	Acquired immunodeficiency syndrome
AMPV	Avian metapneumovirus
ANOVA	A one-way analysis of variance
AOM	Acute otitis media
APV	Avian pneumovirus
ARTI	Acute respiratory tract infection
ATCC	American Type Culture Collection
BAL	Bronchoalveolar lavage
BGM	Buffalo green monkey kidney
BHQ-1	Black Hole Quencher™ 1
BHQ-2	Black Hole Quencher™ 2
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BPD	bronchopulmonary dysplasia
BPIV3	Bovine parainfluenza virus type 3
BRSV	Bovine respiratory syncytial virus
BSC	Biological Safety Cabinet
BSOP	Bacteriology Standard Operating Procedure
BVDV	Bovine viral diarrhoea virus
C	Carboxy
C	Cytosine
$CaCl_2$	Calcium chloride
CCD	charge-coupled device
CDC	Centres for Disease Control and Prevention
cDNA	complementary deoxyribonucleic acid
CDV	Canine distemper virus
CEF	Chicken embryo fibroblasts
CeMV	Cetacean morbillivirus
Cfl	Centre for Infections
CFU	Colony-forming units
CLED	Cystine lactose electrolyte deficient
CMPHL	Clinical Microbiology and Public Health Laboratory
CY-5	Cyanine-5
G-CSF	Granulocyte colony-stimulating factor

CPE	Cytopathic effect
cRNA	Carrier RNA
C_T	Crossing threshold
DALYs	Disability adjusted life years
DDBJ	DNA DataBank of Japan
4-4'DDE	4-4'dichloro-diphenyl-dichloroethane
DEST	Department for Evaluations, Standards and Training
DFA	Direct immunofluorescence assay
DMF	*N, N*-Dimethylformamide
DMV	Dolphin morbillivirus
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide
DoH	Department of Health
dpi	Days post infection
dsRNA	double-stranded RNA
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
EID	Emerging infectious disease
ELISA	Enzyme-linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
EMEM	Eagle's minimal essential medium
ER	Endoplasmic reticulum
ERCs	ECHO 28-rhinovirus-coryzaviruses
EXO	Exonuclease I
6-FAM	6-carboxyfluorescein
F	Fluorophore
F	Fusion
FCS	Foetal Calf Serum
FCV	Feline calicivirus
FITC	Fluorescein isothiocyanate
FRET	Fluorescence resonance energy transfer
G	Glycoprotein
G	Guanine
GBD	Global burden of disease
G-CSF	Granulocyte colony-stimulating factor
GE	Gene end
GM-CSF	Granulocyte-monocyte colony-stimulating factor
GS	Gene start
H	Haemagglutinin
HBoV	Human bocavirus
HCV	Hepatitis C virus
HCl	Hydrochloric acid
HCoV	Human coronavirus

HEp-2	Human laryngeal carcinoma
HepG2	Human hepatoma
HeV	Hendra virus
HIV	Human immunodeficiency virus
HMPV	Human metapneumovirus
HN	Haemagglutinin-neuraminidase
HPA	Health Protection Agency
HPIV	Human parainfluenza virus
HPIV1	Human parainfluenza virus type 1
HPIV2	Human parainfluenza virus type 2
HPIV3	Human parainfluenza virus type 3
HPIV4 A/B	Human parainfluenza virus type 4 A/B
HPLC	High Performance Liquid Chromatography
HPSF	High Purity Salt Free purification technology
HRB	Heptad repeat B
HRSV	Human respiratory syncytial virus
HRV	Human rhinovirus
HRV-C	Human rhinovirus group C
HT-29	Human colon adenocarcinoma
HTLV-I	Human T cell lymphotropic virus type I
HTLV-II	Human T cell lymphotropic virus type II
ICTV	International Committee on the Taxonomy of Viruses
IFA	Indirect immunofluorescence assay
IFN	Interferon
IFN-α	Interferon -alpha
IFN-β	Interferon -beta
IFN-γ	Interferon-gamma
IG	Intergenic region
IHC	Immunohistochemistry
IL-1α	Interleukin-1 alpha
IL-1β	interleukin-1 beta
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
ILI	Influenza-like illness
IOM	Institute of Medicine
IPD	Invasive pneumococcal disease
KC	Mouse IL-8 homologue
kb	Kilobase
L	Large polymerase
LB	Luria-Betani
LED	Light-emitting diode

LLC-MK2	Rhesus monkey kidney
LRTI	Lower respiratory tract infection
M	Matrix
M	Adenine or cytosine
MAb	Monoclonal antibody
$MgSO_4$	Magnesium sulphate
MBL	Mannose-binding lectin
MCL	Maximum composite likelihood
mCMV	Murine cytomegalovirus
MCP-1	Monocyte chemotactic protein 1
MDCK	Madin Darby canine kidney
MEF	Middle ear fluid
MEGA	Molecular Evolutionary Genetics Analysis
MCPyV	Merkel cell polyomavirus
MeV	Measles virus
MFI	Median Fluorescent Intensity
MGB	Minor groove binder
$MgCl_2$	Magnesium chloride
MGP	Magnetic glass particle
MIP-1α	Macrophage inflammatory protein 1α
MOI	Multiplicity of infection
MPV	Murine penumovirus
MRC-5	Normal human foetal lung fibroblast
mRNA	Messenger RNA
MuV	Mumps virus
N	Amino
N	Nucleocapsid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
NCI-H292	Human pulmonary mucoepidermoid carcinoma
NDV	Newcastle disease virus
nvCJD	New variant Creutzfeldt-Jacob disease
NFQ	Non-fluorescent quencher
NiV	Nipah virus
NPA	Nasopharyngeal aspirate
NS1	Non-structural 1
NS2	Non-structural 2
NSG	No significant growth
NSM	National Standard Method
NTC	No template control
ORF	Open reading frame
oRSV	Ovine respiratory syncytial virus

P	Phosphoprotein
PAF	Platelet-activating factor
PAF-R	PAF receptor
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl
PCR	polymerase chain reaction
PCV7	7-valent pneumococcal conjugate vaccine
PCV9	9-valent pneumococcal conjugate vaccine
PCV13	13-valent pneumococcal conjugate vaccine
PDV	Phocine distemper virus
PhHV-1	Phocine herpes virus-1
PFU	Plaque-forming unit
PICU	Paediatric Intensive Care Unit
PLC-PRF5	Human hepatoma
PMV	Porpoise morbillivirus
POCT	Point-of-care testing
PRRSV	Porcine reproductive and respiratory syndrome virus
psi	Per square inch
PTA	phosphotungstic acid
Q	Quencher
QCMD	Quality Control for Molecular Diagnostics
qRT-PCR	quantitative RT-PCR
QSOP	Quality Standard Operating Procedure
R	Adenine or guanine
RAP-PCR	RNA arbitrarily primed PCR
REC	Research Ethics Committee
RD	Human rhabdomyosarcoma
RCGP	Royal College of General Practitioners
ROX	carboxy-X-rhodamine
RMK	Rhesus monkey kidney
RNase	Ribonuclease
RNP	Ribonucleocapsid complex
RNA	Ribonucleic acid
RPM	Revolutions per minute
RPV	Rinderpest virus
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
RR	Relative Risk
RT	Reverse transcription
RTI	Respiratory tract infection
RT-PCR	Reverse transcription polymerase chain reaction
RVP	Respiratory virus panel
SAP	Shrimp Alkaline Phosphatase
SARS	Severe acute respiratory syndrome

SARS-CoV	Severe acute respiratory syndrome coronavirus
SeV	Sendai virus
SH	Small hydrophobic
SIV	Simian immunodeficiency virus
SPU	Sample processing unit
SV-5	Simian virus type 5
SV-41	Simian parainfluenza virus type 41
SVCC	Shell vial centrifugation cultures
T	Thymidine
TAMRA	6-carboxy-tetramethylrhodamine
TBE	Tris-Borate-EDTA buffer
$TCID_{50}$	50% tissue culture infectious dose
TE	Tris-EDTA
TH	T helper
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
T_m	Melting temperature
TM	Transmembrane
tMK	Tertiary monkey kidney
TNAI	Total nucleic acid isolation
TNF-α	Tumor necrosis factor- alpha
Tris	tris(hydroxymethyl)aminomethane
TRTV	Turkey rhinotracheitis
TS	Trichodysplasia spinulosa
TSP	Target-Specific Primer
TSV	TS-associated polyomavirus
TSPE	Target-Specific Primer Extension
UNG	Uracil-N-glycosylase
UTRI	Upper respiratory tract infection
VTM	Virus transport medium
W	Adenine or thymidine
wd-NHBE	well-differentiated normal human bronchial epithelial cells
WHO	World Health Organisation
WRS	Weekly Returns Service
X-GAL	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Y	Cytosine or thymidine
YT	Yeast-Tryptone

CHAPTER ONE

1. Introduction

1.1. The relationship between man and infectious diseases

The relationship between man and infectious diseases is long and turbulent. It has existed since the dawn of civilisation with devastating consequences to susceptible human beings worldwide (Bollet, 1987; Burnet, 1972; Hopkins, 1983; McNeill, 1976; Zinsser, 1935). The early history of infectious diseases was characterised by sudden, unpredictable outbreaks, frequently of epidemic proportion, that ravaged cities, decimated armies and ultimately altered the course of history (Satcher, 1995). The appearance of such manifestations was regarded as a sign of divine displeasure (Scott and Duncan, 2001), a consequence of inciting the wrath of the gods or associated with the configuration of stars or miasmas (Rivers, 1937). Control of many infectious diseases became possible with the pioneering work of Robert Koch and Louis Pasteur and the introduction of the germ theory of disease. With bacteriologic cultivation techniques, came the first isolation and identification of etiologic agents (Satcher, 1995). Identification of the life cycle and reservoir of specific microorganisms, coupled with improvements in food and water safety, hygiene and sanitation, nutrition and housing, and later the discovery of effective antimicrobial therapy and introduction of vaccination and disinfectants, led to major improvements in public health in the late 19th and early 20th century (Satcher, 1995; Cohen 2000; Fauci, 2001). However, declaration of victory against the threat of infectious diseases has befallen to realisation of the enormity of the challenges that lie ahead (Fauci, 2001). The optimism of the 1960s and 1970s has given way to a mature realism that the relationship between human beings and microbes is neither completely predictable nor biased in favour of humans (Zambon and Nicholson, 2003). Indeed, infectious diseases remain rank third as the leading cause of death worldwide (World Health Organisation, 2004). A statistic that serves to reinforce that the history of infectious diseases has been a history of microbes on the march, often in the wake of human beings, and of microbes that have taken advantage of the rich opportunities offered them to thrive, prosper, and spread (Morse, 1995). Indeed, the historical processes that have given rise to the emergence of “new” infections throughout history continue today with unabated force; in fact, they are accelerating, because the conditions of modern life ensure that the factors responsible for disease emergence are more prevalent than ever before (Morse, 1995).

1.2. The Challenge of Emerging and Re-emerging Infectious Disease

The emergence of novel infectious diseases within the past few decades has attracted extensive global attention in both the scientific community and general media. Severe acute respiratory syndrome (SARS) captivated the entire global health community and led to unparalleled international effort coordinated by the World Health Organisation (WHO) (Riley *et al*., 2003) to produce scientific and epidemiologic discoveries with unprecedented speed (Gerberding, 2003) in order to understand and contain the spread of the emerging public health threat. A pre-occupation with hitherto unknown infectious diseases emerged in the aftermath of the SARS epidemic, which at first glance seemed extravagant given the massive immediate health burdens imposed by old scourges such as tuberculosis and malaria (Woolhouse and Gaunt, 2007). An obvious counter argument is the relatively recent advent of human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS), unrecognised less than a generation ago and yet now one of the world's biggest killers (Woolhouse and Gaunt, 2007). AIDS, reinforced by knowledge of other plagues occurring throughout human history, is a reminder that the possibility that novel pathogens could emerge to challenge human progress and survival is real (Morens *et al*., 2004; Woolhouse and Gaunt, 2007). Indeed, it is a paradox that despite technological advancement, human beings remain as vulnerable to new agents as early ancestors were to previous plagues (Hawkey *et al*., 2003).

Emerging infectious diseases (EIDs) are defined as those infections that have newly appeared in a population or have existed but are rapidly increasing in incidence or geographic range (Morse and Schluederberg, 1990; Morse, 1993). New diseases have been emerging at the historically unprecedented rate of one or more per year since the 1970s (WHO, 2007) and this trend is certain to continue. Indeed, more than 100 new or newly identified human pathogens have been identified since 1973 (ProMED, 1994-2001; WHO, 1996, 1998; Institute of Medicine (IOM), 2009; Olano and Walker, 2011; van der Meijden *et al*., 2011). Table 1.1 lists the major diseases or etiologic agents identified during this period.

Table 1.1. Examples of human pathogens recognised since 1973.

Data taken from ProMED, 1994-2001 (**http://fas.org/promed/**); World Health Organisation (WHO), 1996 (**http://www.who.int/whr/1996/en/whr96_en.pdf**); Institute of Medicine (IOM), 2009 (**http://www.iom.edu/Reports/2009/ZoonoticDisease.aspx**); Olano and Walker (2011); van der Meijden *et al*., 2011.

Year	Agent	Year	Agent
2010	Trichodysplasia spinulosa (TS)-associated polyomavirus (TSV)	1991	*Nosema ocularum*
2009	Pandemic (H1N1) 2009 influenza		Guanarito virus
2008	Merkel cell polyomavirus (MCPyV)		*Encephalitozoon hellem*
2007	Human Rhinovirus Group C (HRV-C)		*Ehrlichia chaffeensis*
	KI virus	1990	*Vittaforma corneae*
	WU virus		Trubanaman virus
	Melaka virus		Semliki Forest virus
2005	Human T-lymphotropic virus 4		Reston Ebola virus
	Human T-lymphotropic virus 3		Gan gan virus
	Human coronavirus HKU1		Banna virus
	Human bocavirus	1989	Hepatitis C virus
2004	Human coronavirus NL63		European bat lyssavirus 1
2003	SARS-CoV*		*Corynebacterium amycolatum*
2002	*Cryptosporidium hominis*	1988	Picobirnavirus
2001	*Cryptosporidium felis*		Barmah Forest virus
	Human metapneumovirus	1987	Suid herpevirus 1
	Baboon cytomegalovirus		Sealpox virus
2000	Whitewater Arroyo virus		Dhori virus
1999	TT virus	1986	Rotavirus C
	Nipah virus		Kokobera virus
	Ehrlichia ewingii		Kasokero virus
	Brachiola algerae		Human immunodeficiency virus (HIV)-2
1998	*Trachipleistophora anthropophthera*		Human herpesvirus 6
	Menangle virus		European bat lyssavirus 2
	Brachiola vesicularum		*Cyclospora cayetanensis*
1997	Laguna Negra virus	1985	*Pleistophora ronneafiei*
	Bartonella clarridgeiae		*Enterocytozoon bieneusi*
1996	Usutu virus		Borna disease virus
	Trachipleistophora hominis	1984	*Scedosporium prolificans*
	Metorchis conjunctus		Rotavirus B
	Juquitiba virus		Human torovirus
	Ehrlichia canis	1983	Human immunodeficiency virus (HIV) -1
	New variant Creutzfeldt-Jacob disease (nvCJD)		Human adenovirus F
	Australian bat lyssavirus		Hepatitis E virus
	Andes virus		*Helicobacter pylori*
1995	New York virus		*Capnocytophaga canimorsus*
	Hepatitis G virus		Candiru virus
	Côte d'Ivoire Ebola virus	1982	Seoul virus
	Black creek canal virus		Human T cell lymphotrophic virus type II (HTLV-II)
	Bayou virus		*Escherichia coli* O157:H7
1994	Sabiá virus		*Borrelia burgdorferi*
	Human herpesvirus 8	1981	*Microsporidian africanum*
	Human herpesvirus 7	1980	Puumala virus
	Hendra virus		Human T cell lymphotrophic virus typeI (HTLV-I)
	Anaplasma phagocytophila	1977	Ebola virus
1993	Sin Nombre virus		*Legionella pneumophila*
	Gymnophalloides seoi		Hantaan virus
	Encephalitozoon intestinalis		*Campylobacter* sp.
	Bartonella elizabethae	1976	*Vibrio vulnificus*
1992	Dobrava-Belgrade virus		*Cryptosporidium parvum*
	Bartonella henselae	1975	Parvovirus B19
		1973	Rotavirus

*Severe Acute Respiratory Syndrome-Associated coronavirus (SARS-CoV)

The concept, definitions, and concerns associated with emerging microbial threats were first encapsulated in the landmark publication *Emerging Infections: Microbial Threats to Health in the United States* by the IOM in 1992, which defined the major issues and described the principal causes and mechanisms leading to infectious disease emergence (Mackenzie, 1998). This report provoked immediate response from the US Centres for Disease Control and Prevention (CDC) and WHO and engendered widespread debate within the scientific community (CDC, 1994; Morse, 1995; Satcher, 1995; Truyen *et al*., 1995; CDC, 1998; Ebel and Spielman, 1998; Mackenzie, 1998; Binder *et al*., 1999; Lashley, 2003). Many diverse factors contribute to the emergence or re-emergence of infectious diseases (Smolinski *et al*., 2003) (Table 1.2). These factors are not mutually exclusive and indeed several factors may contribute to emergence of a disease (Morse, 1995).

Table 1.2. Factors in infectious disease emergence.

Microbial adaptation and change	Technology and industry
Human susceptibility to infection	Breakdown of public health measures
Climate and weather	Poverty and social inequality
Changing ecosystems	War and famine
Human demographics and behaviour	Lack of political will
Economic development and land use	Intent to harm
International travel and commerce	

In common with the IOM exists a growing recognition that zoonotic infectious agents have provided a key element for the emergence of infectious disease episodes in humans (Morse, 1993, 1995; Osburn, 1996; Chomel, 1998; Murphy, 1998; Palmer *et al*., 1998; Daszak *et al*., 2000). History is rich with examples of infections that originated as zoonoses suggesting that the "zoonotic pool" – introductions of viruses from other species – is an important and potentially rich source of emerging disease while periodic discoveries of "new" zoonoses suggest that the zoonotic pool is by no means exhausted (Morse, 1995). Indeed, a systemic literature review conducted in 2001 identified 1415 species of infectious organism known to be pathogenic to humans. Of these species, 868 (61%) were zoonotic. Whilst among emerging pathogens, 175 species (12%) were associated with disease in humans and of these 132 (75%) were zoonotic (Taylor *et al*., 2001). An updated version of this review was generated in 2005 (Woolhouse and Gowtage-Sequeira, 2005) and more recently in 2007 to provide a more complete picture of new species of human pathogen (Woolhouse and Gaunt, 2007). The latter review encompasses human pathogen species discovered since 1980 and

suggests that 1399 species of human pathogen exist (Woolhouse and Gaunt, 2007). The slight variation that exists between each review with regard to the number of species of human pathogen identified reflects changes in taxonomy and discoveries of previously unknown pathogens (Woolhouse and Gowtage-Sequeira, 2005) as well as the methodology used to perform each review. However, despite these variations it is clear that viruses triumph as emerging human pathogens. Indeed, viruses comprise the largest group of emerging human pathogens despite representing only a small fraction of all recognised human pathogen species (Woolhouse and Gaunt, 2007). Reports of novel virus species in humans are occurring at a rate of over two per year, a rate that exceeds that of any other group of pathogens (Woolhouse and Gaunt, 2007). However, the human race is not alone in its conflict with infectious menaces that threaten public health. Emerging infectious disease of wildlife poses a substantial threat to global biodiversity (Daszak *et al*., 2000). Historically, wildlife diseases were considered important only when the health of economically important domestic species or humans was threatened (Daszak *et al*., 2000). However, infectious disease in wildlife species is now recognised as a substantial global threat to be taken in earnest (Harvell *et al*., 1999; Daszak *et al*., 2000; Daszak *et al*., 2001; Williams *et al*., 2002; Bataille *et al*., 2009; Blehert *et al*., 2009; Kilpatrick *et al*., 2010; Robinson *et al*., 2010).

1.3. The *Paramyxoviridae*: An Expanding Family of Important Viral Pathogens

The family *Paramyxoviridae* includes some of the great and ubiquitous diseasing causing viruses (Lamb and Parks, 2007). These viruses are responsible for some of the most severe and contagious diseases of susceptible human beings and domestic and wildlife species worldwide including measles, canine distemper, and two economically important diseases of livestock: rinderpest, in bovine species, and peste des petits ruminants, in goats and sheep. The importance and breadth of this virus family has increased during the last two decades as new viruses have emerged (Dutch, 2010) both fortuitously and as a consequence of investigations subsequent to disease outbreaks (Wang and Eaton, 2001). The host range of viruses within the family *Paramyxoviridae* is typically limited and crossover events rare (Virtue *et al*., 2009). However, several new viruses have emerged as a result of spill-over events in wild species that are associated with a number of severe diseases of humans and animals (Moreno-López *et al*., 1986; Murray *et al*., 1995; Selvey *et al*., 1995; Philbey *et al*., 1998; Barrett, 1999, Chua *et al*., 2000, Di Guardo, *et al*., 2005). These viruses, including canine distemper virus, Nipah and Hendra virus have proven an exception, displaying high virulence and a wide host range (Virtue *et al*., 2009). The propensity of a number of other

new viruses to cause human disease is unknown (Virtue *et al*., 2009). However, given the genetic similarity to known zoonotic paramyxoviruses the potential exists for them to cross the species barrier into new hosts including humans (Virtue *et al*., 2009).

1.3.1. Canine Distemper Virus

Canine distemper virus (CDV) is the causative agent of a lethal infectious disease of the domestic dog (*Canis familiaris*) that has been recognised for more than 200 years (Appel, 1991). The host spectrum of CDV is broad and all families in the order Carnivora are susceptible to infection (Williams, 2001). The importance of this disease has increased with the expansion of the host range and the dramatic emergence of large-scale epizootics of canine distemper in captive (Appel, *et al*., 1994) and free-ranging felids (Harder *et al*., 1995; Kock *et al*., 1998; Morell, 1994; Roelke-Parker *et al*., 1996) fuelled by multiple spill-overs from sympatric carnivore species (Craft *et al*., 2009) and vaccine-induced infections in a wide variety of species (Bush *et al*., 1976; Carpenter *et al*., 1976; Bush and Roberts, 1977; Halbrooks *et al*., 1981; Itakura *et al*., 1979; Kazacos *et al*., 1981; Sutherland-Smith *et al*., 1997). However, most crucial is the direct threat that the disease presents to the persistence of endangered susceptible wildlife species (Anderson, 1995) and the unexpected emergence of canine distemper in species with no known natural susceptibility to the virus including the collared peccary (*Tayassu tajacu*) (Appel *et al*., 1991; Noon *et al*., 2003), Japanese monkeys (*Macaca fuscata*) (Yoshikawa *et al*., 1989), rhesus monkeys (*Macaca mulatta*) (Sun *et al*., 2010), (Barrett *et al*., 2004), Baikal seals (*Phoca siberica*) in Siberia (Grachev *et al*., 1989; Osterhaus *et al*., 1989; Mamaev *et al*., 1996) and Caspian seals (*Phoca caspica*) (Kennedy *et al*., 2000) in the Caspian Sea.

1.3.2. Morbillivirus Infections in Aquatic Mammals

Epizootics of infectious disease attributed to infections with three novel members of the genus *morbillivirus*: phocine distemper virus (PDV), porpoise morbillivirus (PMV) and dolphin morbillivirus (DMV) has lead to mass die-offs of several free-living pinniped and cetacean populations around the world (Di Guardo *et al*., 2005). These include harbour seals (*Phoca vitulina*) and grey seals (*Halichoerus grypus*) in Europe, (Kennedy *et al*., 1988; Osterhaus and Vedder, 1988; Osterhaus *et al*., 1988), Mediterranean monk seals (*Monachus monachus*) off the coast of Mauritania (Osterhaus *et al*., 1998), striped dolphins (*Stenella coeruleoalba*) in the Mediterranean Sea (Di Guardo *et al*., 1992**;** Domingo *et al*., 1990, 1992; Raga *et al*., 2008; Van Bressem *et al*., 1991, 1993), bottlenose dolphins (*Tursiops truncatus*) along the United

States coast (Krafft *et al*., 1995; Lipscomb *et al*., 1994a; 1994b; 1996; Shulman *et al*., 1997; Taubenberger *et al*., 1996), common dolphins in the black sea (Birkun *et al*., 1999) and long finned pilot whales (*Globicephalus melas*) (Fernández *et al*., 2008). Among the still debated or even controversial issues regarding morbillivirus infection in sea mammals, the one related to the origin of the causative agents remains a particular concern (Di Guardo *et al*., 2005). It is suggested that other species of marine mammal including pilot whales (*Globicephala melas*) and harp seals (*Phoca groenlandica*) acted as reservoirs of infection while outbreaks of disease were triggered or influenced by the synergistic interaction of multiple environmental factors (Di Guardo *et al*., 2005). These include a number of environmental pollutants with special emphasis on certain organochlorine compounds such as polychlorinated biphenyls (PCBs), dioxins, and 4-4'dichloro-diphenyl-dichloroethane (4-4'DDE) as well as on heavy metals (Di Guardo *et al*., 2005).

1.3.3. Paramyxoviruses in Bats

Bats are the natural reservoir for six recognised paramyxoviruses: Hendra and Nipah virus, the only known members of the new genus, *Henipavirus*, as well as Menangle virus, Tioman virus, bat parainfluenza virus, and Mapuera virus (Breed, 2008). At least three of these are able to infect humans and domestic animals. Ever-increasing human encroachment on natural habitats, combined with the ability of some bats to adapt to anthropogenic environmental changes has led to increased contact between bats, domestic animals and humans (Breed, 2008).

1.3.4. Other Emerging Paramyxoviruses

A number of other emerging paramyxoviruses remain unclassified within the family *Paramyxoviridae* by the International Committee on the Taxonomy of Viruses (ICTV) and the propensity of these viruses to cause human disease is unknown (Virtue *et al*., 2009) (Table 1.6). Although these viruses have yet to be identified as causing human disease, the potential for cross-species transmission exists and genetic diversity in the viral genomes may provide clues as to the risk of zoonoses (Virtue *et al*., 2009). The emergence of new paramyxoviruses provides a unique opportunity to study novel and important diseases, extend knowledge of paramyxoviruses, and appreciate the diversity of viruses in this important family (Wang and Eaton, 2001).

Table 1.3. Paramyxoviruses of unknown zoonotic potential.

Virus	Genus	Year	Source of Isolation	Host Range	Reference
Nariva virus	Unclassified	1960s	Short-tailed Cane Mouse (*Zygodontomys B. Brevicauda*), Eastern Trinidad	Mice	Tikasingh *et al*., (1966)
Tupaia paramyxovirus	Unclassified	1970s	Southeast Asian tree shrew (*Tupaia belangeri*), Thailand	Tree Shrew	Tidona *et al*., (1999)
Mossman virus	Unclassified	1970s	Bush Rat (*Rattus fuscipes*) & Cape York Rat (*Rattus leucopus*), Australia	Rats	Campbell *et al*., (1977)
J virus	Unclassified	1972	Moribund mice (*Mus musculus*), Australia	Mice	Jun *et al*., 1977; Mesina *et al*., (1974)
Fer-de-Lance virus	Unclassified	1972	Fer-de-Lance viper (*Bothrops atrox*), Switzerland	Snakes	Fölsch, D. W., P. Leloup. (1976)
Mapuera virus	*Rubulavirus*	1979	Little Yellow-shouldered Bat (*Sturnira lilium*), Brazil	Bats	Zeller *et al*., (1989)
Salem virus	Unclassified	1992	Horses, USA	Horses	Renshaw *et al*., (2000)
Atlantic salmon paramyxovirus	Unclassified	1995	Atlantic salmon (*Salmo salar* L.), Norway	Fish	Kvellestad *et al*., (2003)
Beilong virus	Unclassified	2005	Human mesangial cells, China	Rodents (?)	Li *et al*., (2006)
Tuhoko virus 1, 2, 3	Unclassified	2000s	Leschenault's Rousette (*Rousettus leschenaultii*), China	Bats	Lau *et al*., (2010)

Adapted from Virtue *et al*., (2009).

1.4. Human metapneumovirus

1.4.1. Discovery of a New Virus

1.4.1.1. Virus isolation and characterisation

Over a 20-year period, researchers in the Netherlands identified a virus in nasopharyngeal aspirate (NPA) samples taken from 28 epidemiologically unrelated children suffering from respiratory tract infection (RTI) (van den Hoogen *et al*., 2001). The cytopathic effect (CPE) induced by this virus on cultured cells was virtually indistinguishable from that caused by human respiratory syncytial virus (HRSV) with characteristic syncytia formation followed by the rapid internal disruption and subsequent detachment of cells from the monolayer (van den Hoogen *et al*., 2001). However, routine diagnostic studies performed by a combination of direct immunofluorescence assay (DFA) and indirect immunofluorescence assay (IFA) failed to detect common viral agents of RTI including HRSV, human parainfluenza virus (HPIV) types 1-3 and influenza virus types A and B (van den Hoogen *et al*., 2001). Negative contrast electron microscopy revealed the presence of pleomorphic particles in the range of 150-600 nm, with short envelope projections in the range of 13-17 nm while nucleocapsids were rarely observed, suggestive of a paramyxovirus (van den Hoogen *et al*., 2001) (Figure 1.1). Furthermore, consistent with the biochemical properties of enveloped viruses including the *Paramyxoviridae*, standard chloroform treatment resulted in a 10,000-fold reduction in infectivity, infected cell culture supernatant did not display haemagglutinating activity with turkey, chicken or guinea pig erythrocytes and virus replication was dependent on trypsin in cell cultures. These combined virological data indicated that this earlier undiscovered virus was a member of the family *Paramyxoviridae* (van den Hoogen *et al*., 2001).

Reverse transcription polymerase chain reaction (RT-PCR) analyses using specific primer sets for known paramyxoviruses including HPIV types 1-4, mumps virus (MuV), measles virus (MeV), HRSV, simian virus type 5 (SV-5), Sendai virus (SeV) and Newcastle disease virus (NDV) were performed at low stringency in order to detect potentially related viruses. However, the virus-specific primers failed to amplify a genomic signal indicating that this new virus was distinct from those viruses for which the primer sets had been selected. Furthermore, virus-specific antisera raised in experimental ferrets and guinea pigs did not react in IFA with cells infected with a panel of paramyxoviruses and orthomyxoviruses (van den Hoogen *et al*., 2001). The identity of this agent remained elusive.

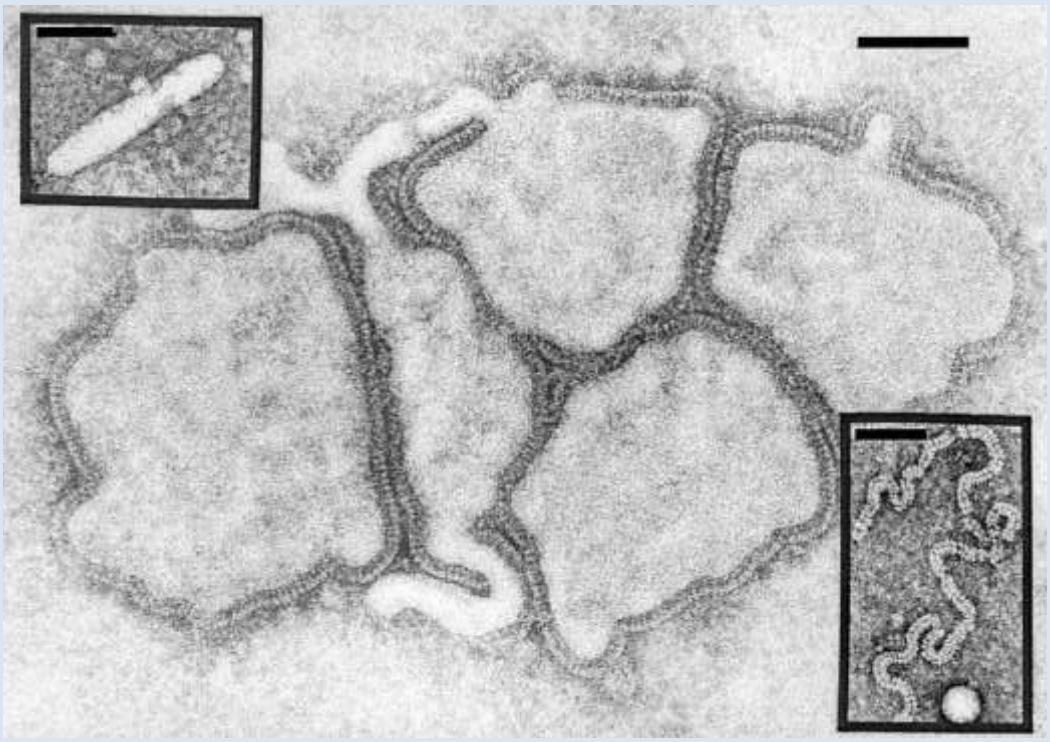


Figure 1.1. Negative-stain electron micrographs of human metapneumovirus.
The centre image shows 5 pleomorphic HMPV particles; note the projections along the periphery of the viruses. The upper left and lower right insets show the nucleocapsid and filamentous rod-like particle, respectively. Staining was done with 2% phosphotungstic acid (PTA). Bar markers represent 100 nm (Peret et al., 2002).

1.4.1.2. Ribonucleic acid arbitrarily primed polymerase chain reaction

Identification of this newly discovered virus was accomplished by the generation of simple and reproducible ribonucleic acid (RNA) fingerprints of the complex viral genome using short primers of arbitrary nucleotide sequence and the polymerase chain reaction (PCR) (Welsh and McClelland, 1990). The method, RNA arbitrarily primed PCR (RAP-PCR), involves two cycles of low stringency complementary deoxyribonucleic acid (cDNA) synthesis followed by PCR amplification at higher stringency to amplify the products (Welsh and McClelland, 1990; Welsh *et al*., 1992). No prior sequence information is required. Instead, this method uses primers chosen without regard to the sequence of the genome to be fingerprinted (Welsh and McClelland, 1990). First strand cDNA synthesis by reverse transcription is initiated from an arbitrarily chosen primer at sites in the RNA that best match the primer. Second-strand synthesis is initiated by extension of the same arbitrarily primer site at sites of adequate match on the first-strand cDNA product by using *Taq* polymerase. The products of cDNA synthesis serve as templates for high stringency PCR and displayed on a gel as a fingerprint

representing between 10 and 50 RNA molecules, depending on the choice of arbitrary primer. Any differences in the pattern produced by a primer in different RNA populations reflect abundance differences in individual RNAs. Many fingerprints can be displayed on a single gel, allowing the simultaneous comparison of abundances for several hundred RNAs (Ralph *et al*., 1993). To this end, van den Hoogen *et al*. (2001) infected tertiary monkey kidney (tMK) cells with either the novel virus or HPIV type-1 as a control. Propagated virus was concentrated from the supernatant by ultracentrifugation on a 20-60% sucrose gradient. The gradient fractions were then inspected for the presence of virus particles by electron microscopy and polyacrylamide gel electrophoresis followed by silver staining. Only fractions that appeared to contain nucleocapsids were retained for RNA isolation and RAP-PCR. Twenty differentially displayed fragments specific for the unidentified virus were purified, cloned, and sequenced. Comparison of nucleotide and amino acid (aa) sequences with published sequences in the GenBank® genetic sequence database using the Basic Local Alignment Search Tool (BLAST) software (www.ncbi.nlm.nih.gov/BLAST) revealed 10 fragments displayed resemblance to avian metapneumovirus (AMPV), also known as avian pneumovirus (APV) and turkey rhinotracheitis (TRTV), the etiological agent of a highly contagious upper respiratory disease in turkeys and swollen head syndrome in chickens (Cook, 2000). These 10 fragments were located in the genes coding for the nucleocapsid (N) protein, the matrix (M) protein, the fusion (F) protein and the large polymerase (L) protein (van den Hoogen *et al*., 2001). Completion of sequence information for the 3' end of the viral genome was achieved by designing primers based on the partial sequence information available for isolate 00-1 identified from the RAP-PCR fragments, and published leader and trailer sequences for the *Pneumovirinae* (van den Hoogen *et al*., 2001). Analysis of the sequences of these fragments revealed the absence of open reading frames (ORFs) of the non-structural proteins NS1 and NS2 at the extreme of the 3' end of the viral genome and positioning of the putative F-ORF immediately adjacent to the putative M-ORF. In contrast to the genomic organisation of pneumoviruses, metapneumoviruses lack NS1 and NS2 genes while different genes occupy a position between genes M and L. The absence of ORFs between the M and F genes and the lack of NS1 and NS2 genes adjacent to the N gene in this new virus was consistent with the genomic arrangement of AMPV. Furthermore, genetic analysis of the translated sequences for the putative N, M, P, and F genes revealed that this virus displayed a high percentage of sequence homology to the recently proposed genus *Metapneumovirus* (average of 66%) as compared to the genus *Pneumovirus* (average of 30%). This agreement led to the provisional classification of this novel virus as the first mammalian member of the genus *Metapneumovirus* and was named human

metapneumovirus (HMPV) (van den Hoogen *et al*., 2001). Formal classification of this virus within the subfamily *Pneumovirinae* and the genus *Metapneumovirus* was approved by the ICTV in 2005 (Fauquet *et al*., 2005). The *Pneumovirinae* are represented by the genera *Pneumovirus* and *Metapneumovirus* (Fauquet *et al*., 2005) (Figure 1.2).

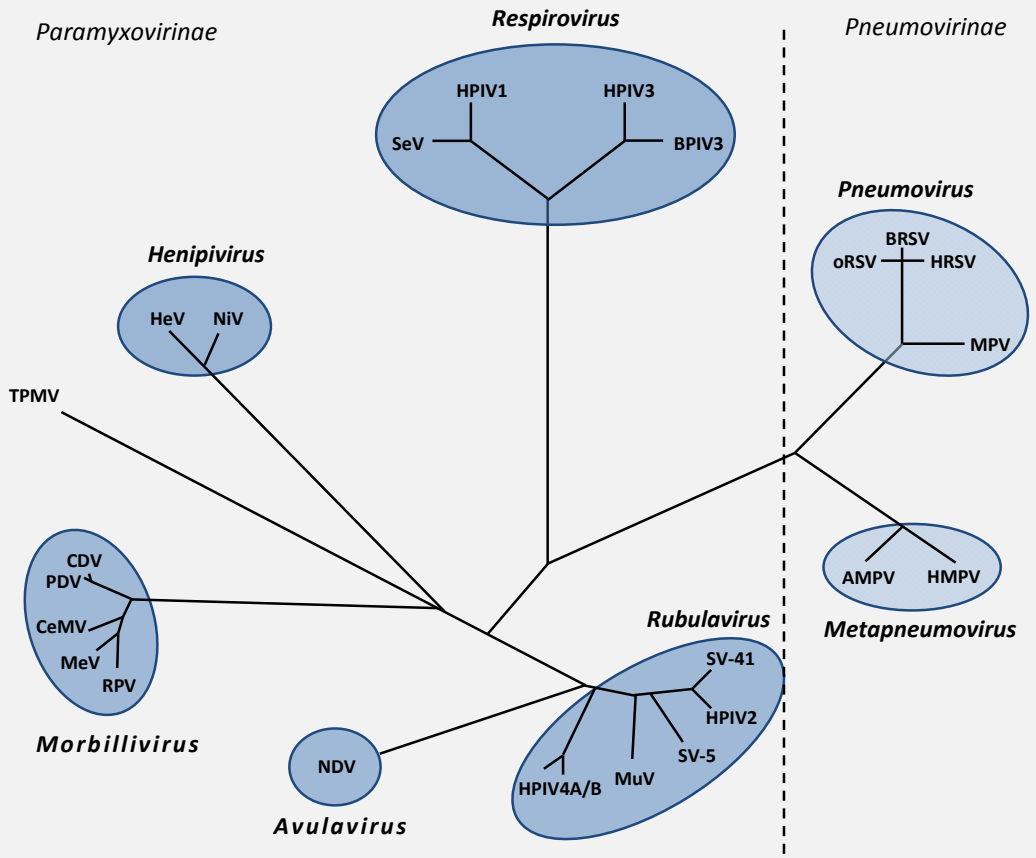


Figure 1.2. Evolutionary relationships of the family *Paramyxoviridae*.

Phylogenetic majority-rule consensus tree based on a combined cluster alignment of nucleocapsid and phosphoprotein amino acid sequences of selected family members. Viruses are grouped according to genus and abbreviated as follows: RPV: rinderpest virus, MeV: measles virus, CeMV: cetacean morbillivirus, PDV: phocine distemper virus, CDV: canine distemper virus; HeV: Hendra virus, NiV: Nipah virus; HPIV1: human parainfluenza virus type 1, HPIV3: human parainfluenza virus type 3, SeV: Sendai virus, BPIV3: bovine parainfluenza virus type 3; NDV: Newcastle disease virus; MuV: Mumps virus, HPIV2: human parainfluenza virus type 2, HPIV4A/B: human parainfluenza virus type 4 A/B, SV-5: simian parainfluenza virus type 5, SV-41: simian parainfluenza virus type 41; oRSV: ovine respiratory syncytial virus, BRSV: bovine respiratory syncytial virus, HRSV: human respiratory syncytial virus, MPV: murine pneumonia virus; AMPV: avian pneumovirus, HMPV: human metapneumovirus.

1.4.2. Anonymous until now…but why?

Analysis of human serum samples collected in 1958 revealed that HMPV has been widespread in the human population for at least 50 years (van den Hoogen *et al*., 2001). It is therefore not surprising that the question has arisen why this increasingly important respiratory pathogen has remained elusive until now, especially in an era of advanced molecular technology (Domachowske, 2003). Van den Hoogen *et al*., (2001) attribute the anonymity of HMPV in part to its poor replication in continuous cell lines that are used by many diagnostic virology laboratories for virus isolation. Second, HMPV displays very slow replication kinetics *in vitro* in contrast to other human respiratory viruses. Indeed, van den Hoogen *et al*., (2001) reported cytopathic effects were visible 10-14 days post-inoculation while Boivin *et al*., (2002) noted that cytopathic effects were apparent after an incubation time of between 3-23 days (mean 17.3 days). Chan *et al*., (2003) observed cytopathic effects after 10 to 22 days incubation and suggest prolonged incubation to 28 days may improve the sensitivity of detection. Third, replication of HMPV *in vitro* appeared to be trypsin dependent and many of the earlier studies to find respiratory pathogens did not use trypsin. Finally, HMPV exhibits low nucleotide sequence homology with other members of the family *Paramyxoviridae* allowing HMPV to escape detection under low-stringency PCR conditions dependent on cross-reactivity with primers designed to amplify known viral sequences (van den Hoogen *et al*., 2001). Domachowske *et al*., (2003) suggest the most common reason for "false-negative" viral diagnostic evaluations is sub-optimal collection and processing of clinical samples. Ideally, samples collected for viral cultures should be obtained by nasal wash or nasal aspirate, placed on ice for transport to the clinical virology laboratory, freshly inoculated onto appropriate cell monolayers, and incubated at 33°C. Respiratory viral pathogens, particularly pneumoviruses, are exquisitely temperature sensitive. HRSV for example becomes increasingly difficult to culture from clinical samples following overnight refrigeration or a single cycle of freeze-thawing. Even when they are processed and stored in an optimal fashion, rescuing pneumoviral isolates from clinical samples becomes increasingly difficult over time. However, of worth noting is the observation by van den Hoogen *et al*., (2001) of the CPE induced by HMPV in tMK cells. In the initial report, van den Hoogen *et al.,* (2001) observed that the cytopathic effects induced by HMPV are virtually indistinguishable from HRSV, with characteristic syncytia formation followed by the rapid internal disruption and subsequent detachment of cells from the monolayer. However, other studies, notably those of Boivin *et al*., (2002) and Chan *et al*., (2003), failed to replicate the findings of the original study. These latter studies identified a CPE characterised by small, round, refringent

cells, without syncytia formation in most cases. The CPE progressed slowly to detachment from the cell monolayer (Chan *et al*., 2003). Furthermore, all studies to date have isolated HMPV at 37°C. However, isolation of respiratory viruses requires incubation at 33°C. The failure of these studies to confirm a consistent CPE may relate to the genetic variation between HMPV isolates. Van den Hoogen *et al*., (2004a) have identified that viruses genetically related to the prototype strain NL/1/100 (serotype A) revealed cytopathic effects more clearly on tMK cells than viruses related to prototype strain NL/1/99 (serotype B). Indeed, in search of a cell line similarly susceptible for both types a subclone of Vero cells was generated. Vero cell clone 118 was permissive for infection with viruses from all four lineages, and cytopathic effects were easily observed (van den Hoogen *et al*., 2004a).

1.4.3. Virus Structure and Genome Organisation

The subfamily *Pneumovirinae* and in particular HRSV and AMPV have provided substantial insight into the structure and genomic organisation of HMPV. The virion consists of a helical nucleocapsid contained within a lipid bilayer envelope that is derived from the plasma membrane of the host cell (Easton *et al*., 2004) (Figure 1.3). Inserted into the envelope of all members of the subfamily *Pneumovirinae* are three transmembrane surface glycoproteins, an attachment glycoprotein (G) that differs from haemagglutinin-neuraminidase (HN) and haemagglutinin (H) attachment proteins in that it has neither haemagglutination nor neuraminidase activity, a F protein, and a small hydrophic (SH) protein (Bossart and Broder, 2011; Easton *et al*., 2004). Inside the envelope is the approximately 13,000 nucleotide single-stranded negative sense RNA genome that is encapsidated with the N protein and contains eight genes in the order 3'-N-P-M-F-M2-SH-G-L-5' that encode nine different putative proteins (van den Hoogen *et al*., 2002; Biacchesi *et al*., 2003) analogous to HRSV (Figure 1.4) and AMPV. The N protein and the genome RNA form the ribonuclease (RNase) resistant nucleocapsid core to which the phosphoprotein (P) and L protein are attached (Lamb and Parks, 2007). This complex of proteins termed the ribonucleocapsid complex (RNP) has RNA-dependent RNA transcriptase activity and initiates intracellular virus replication (Lamb and Parks, 2007). In addition, the genome contains non-coding 3' leader and 5' trailer sequences and intergenic regions (Herfst *et al*., 2004). The viral promoter is contained within the 3'-terminal 57 nucleotide of the genome (Biacchesi *et al*., 2004a).

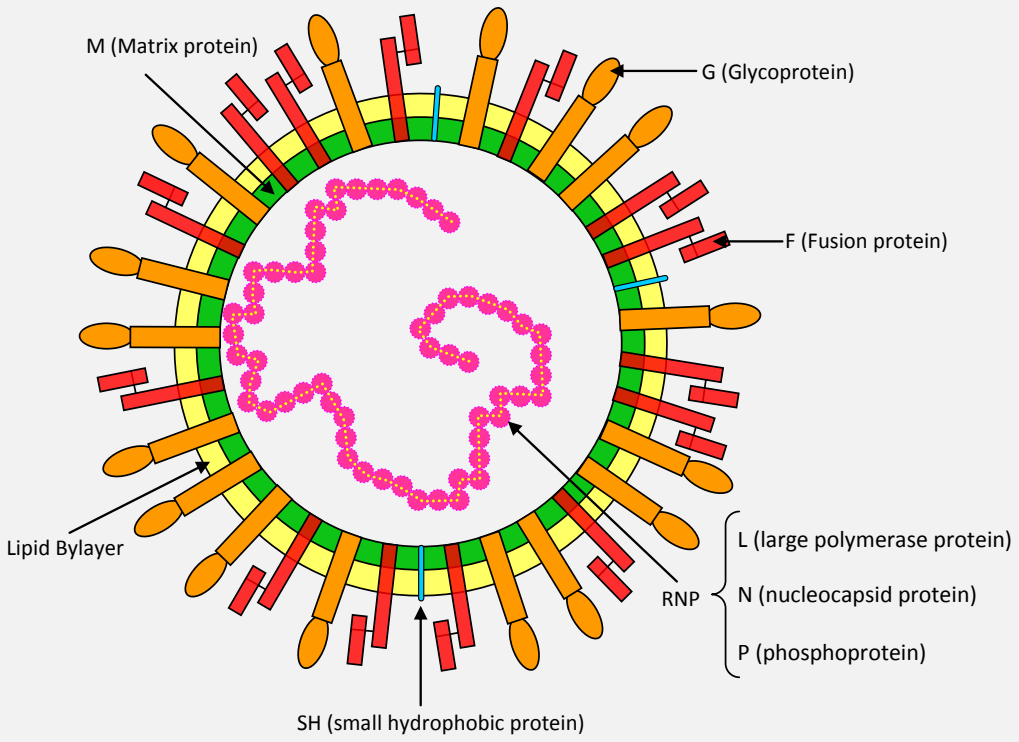
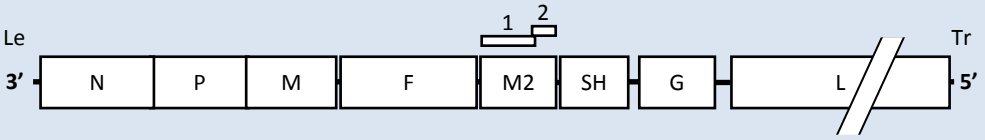


Figure 1.3. Schematic representation of the structure of members of the *Pneumovirinae*. Embedded in the lipid membrane are the attachment (G) glycoprotein, fusion (F) protein and small hydrophobic (SH) protein. Inside the virus is the negative-stranded virion RNA that is encapsidated with the nucleocapsid protein (N). The phosphoprotein (P) and large polymerase (L) protein are associated with the N protein and together this complex has RNA-dependent RNA transcriptase activity. The idealised illustration is adapted from Lamb and Parks (2007).

Residing between the envelope and the core is the viral M protein that is highly hydrophobic in character. This protein is important in virion architecture and is released from the core during virus entry (Lamb and Parks, 2007). The M2 gene is unique to members of the subfamily *Pneumovirinae* (van den Hoogen *et al*., 2002). The M2 gene of HMPV contains two overlapping ORFs that encode 2 proteins, M2-1, and M2-2 (Buchholz *et al*., 2005). Evidence suggests that M2-1 ORF is dispensable for virus recovery and replication *in vitro*. This presents a sharp contrast to HRSV M2-1 that is essential for viral transcription and replication (Buchholz *et al*., 2005). However, expression of M2-1 was required for detectable replication *in vivo* (Buchholz *et al*., 2005). M2-2 also was dispensable for growth *in vitro* but expression of the protein appears to have an authentic role in regulating RNA synthesis (Buchholz *et al*., 2005; Kitagawa *et al*., 2010) and maintaining genetic stability of the HMPV genome (Schickli *et al*., 2008). Thus, HMPV accessory protein M2-2 may be also a multifunctional protein (Kitagawa *et al*., 2010).

Genus *Metapneumovirus*: Human metapneumovirus



Genus *Pneumovirus*: Human respiratory syncytial virus

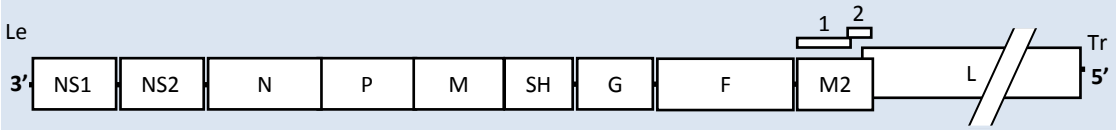


Figure 1.4. Comparison of the genomic maps (not to scale) and encoded proteins of respiratory syncytial virus and human metapneumovirus positioned to align the 5' end of the genome.

Genes are represented as rectangles identified according to the encoded protein; in the case of M2, the overlapping open reading frames (ORFs) are illustrated and identified. HRSV encodes eleven separate proteins. In contrast to the genomic organisation of pneumoviruses, metapneumoviruses lack NS1 and NS2 genes while different genes occupy a position between genes M and L. Abbreviations: Le: Leader, NS1 and NS2: non-structural proteins 1 and 2, N: nucleoprotein, P: phosphoprotein, M: matrix protein, SH: small hydrophobic protein, G: attachment protein, F: fusion protein, M2-1: M2 OFR1 protein, M2-2: M2 ORF2 protein; L: large polymerase protein, Tr: trailer. M2-1 and M2-2 proteins of *Pneumovirinae* are regulatory proteins. M2-1 functions as a transcription elongation factor and M2-2 as a RNA synthesis regulatory factor in HRSV. Evidence suggests M2-2 is also essential for efficient HMPV replication but the function of M2-1 is not completely understood as M2-1 is not essential for virus viablity. The idealised illustration is adapted from Collins and Crowe (2007).

1.4.3.4. The attachment glycoprotein

The organisation of the HMPV G protein is consistent with that of an anchored type II transmembrane protein although the G protein shares no discernible sequence identity at the nucleotide or aa level with other virus genes or gene products (van den Hoogen *et al*., 2002). The amino (N)-terminus contains a hydrophilic cytoplasmic domain followed by a single short hydrophobic region and a mainly hydrophilic extracellular carboxy (C)-terminus (van den Hoogen *et al*., 2002). The high content of serine and threonine residues, potential sites for *O*-glycosylation in combination with an unusual abundance of proline residues confirms a heavily glycosylated mucin-like structure for the HMPV G protein (Peret *et al*., 2004), which is consistent with HRSV and AMPV (van den Hoogen *et al*., 2002). The finding that the HMPV G protein is synthesised as an N-glycosylated intermediate form and subsequently processed to a mature form, which contains extensive *O*-linked carbohydrates, provides authentication of initial predictions of the structural features of the HMPV G protein that were made solely on the basis of sequence analysis (Liu *et al*., 2007a).

1.4.3.2. The fusion protein

Analysis of the aa sequence of the HMPV F protein revealed 81% sequence identity with AMPV-C, 67% with AMPV-A and -B, 33–38% with other pneumovirus F proteins, and only 10–18% with other members of the *Paramyxoviridae* (van den Hoogen *et al*., 2002). Despite the low sequence homology with other members of the family *Paramyxoviridae*, the F glycoprotein of HMPV appears to be similar in overall structure and function to those of the more highly characterized prototypic members *Paramyxovirinae*, such as NDV and SeV (Biacchesi *et al*., 2006). The prototypic *Paramyxoviridae* F protein is a type I integral membrane protein that spans the membrane once and contains an N-terminal cleaved signal peptide and a C-proximal hydrophobic transmembrane (TM) domain that anchors the protein in the membrane leaving a short cytoplasmic tail that extends into the cytoplasm (Lamb and Parks, 2007; Miller *et al*., 2007).

1.4.3.3. The small hydrophobic protein

The HMPV SH protein, similar to its HRSV counterpart is a predicted type II integral membrane protein that is anchored by a hydrophobic signal/anchor sequence proximal to the hydrophilic N-terminus and a hydrophilic extracellular C-terminus (Biacchesi *et al*., 2003; van den Hoogen *et al*., 2002). The SH protein of HMPV is the largest known to date among the *Pneumoviridae* (179 aa for the Canadian HMPV isolate CAN97-83, versus 174 aa for AMPV A, 81 aa for BRSV, and 64 aa for HRSV A) (Biacchesi *et al*., 2003; van den Hoogen *et al*., 2002). The function of the SH protein is not completely understood but a potential role in regulating host immune responses is suggested (Bao *et al*., 2008).

1.4.4. The Replication Strategy of the Paramyxoviridae

1.4.4.1. Viral Adsorption and Entry

All aspects of the replication of *Paramyxoviridae* occur in the cytoplasm (Lamb and Parks, 2007) (Figure 1.5). The process commences upon adsorption of the virus to the cellular receptor on the plasma membrane of a receptive host cell at the neutral pH found at the cell surface. Entry into target cells is mediated by the attachment glycoprotein (G, H or HN) and the F protein which is responsible for fusion between the virion envelope and the host cell plasma membrane (Smith *et al*., 2009). However, the attachment protein G of members of the subfamily *Pneumovirinae* may not be obligatory for attachment and viral entry in all cases (Smith *et al*., 2009). Indeed, recombinant HMPV lacking the viral attachment glycoprotein (ΔG) can enter cells and proliferate *in vitro* (Biacchesi *et al*., 2004b; Biacchesi *et al*., 2005) and *in vivo* in a permissive nonhuman primate host, the African green monkey (*Cercopithecus aethiops*) (Biacchesi *et al*., 2005). Thus, it appears that the HMPV F protein alone is sufficient to mediate attachment and fusion in the absence of other surface proteins (Biacchesi *et al*., 2004b). Recent evidence suggests that the HMPV F protein engages αvβ1 integrin receptor, a heterodimeric cell-surface molecule, as a functional receptor to mediate virus entry (Cseke *et al*., 2009). These findings also raise the important question of what triggers the F protein to initiate fusion since interactions with the attachment protein clearly do not control fusion initiation (Dutch, 2010). In fact, exposure to low pH triggers membrane fusion mediated by the HMPV F protein (Schowalter *et al*., 2006) while electrostatic repulsion in the heptad repeat B (HRB) linker region contributes to the triggering process (Schowalter *et al*., 2009). However, it is unlikely that exposure to low pH is a general trigger of the HMPV F protein for membrane fusion (Herfst *et al*., 2008). Recent examination of the biological significance of low pH fusion in virus entry suggests that HMPV utilises the endocytic entry mechanism (Schowalter *et al*., 2009). The low pH environment encountered after the endocytosis of HMPV may be an important physiological trigger of the F conformational change that results in subsequent membrane fusion (Schowalter *et al*., 2009). Endosomal entry could offer protection to viruses from the host immune system and in combination with lowered pH provide unique environments that assist in productive infection (Smith *et al*., 2010).

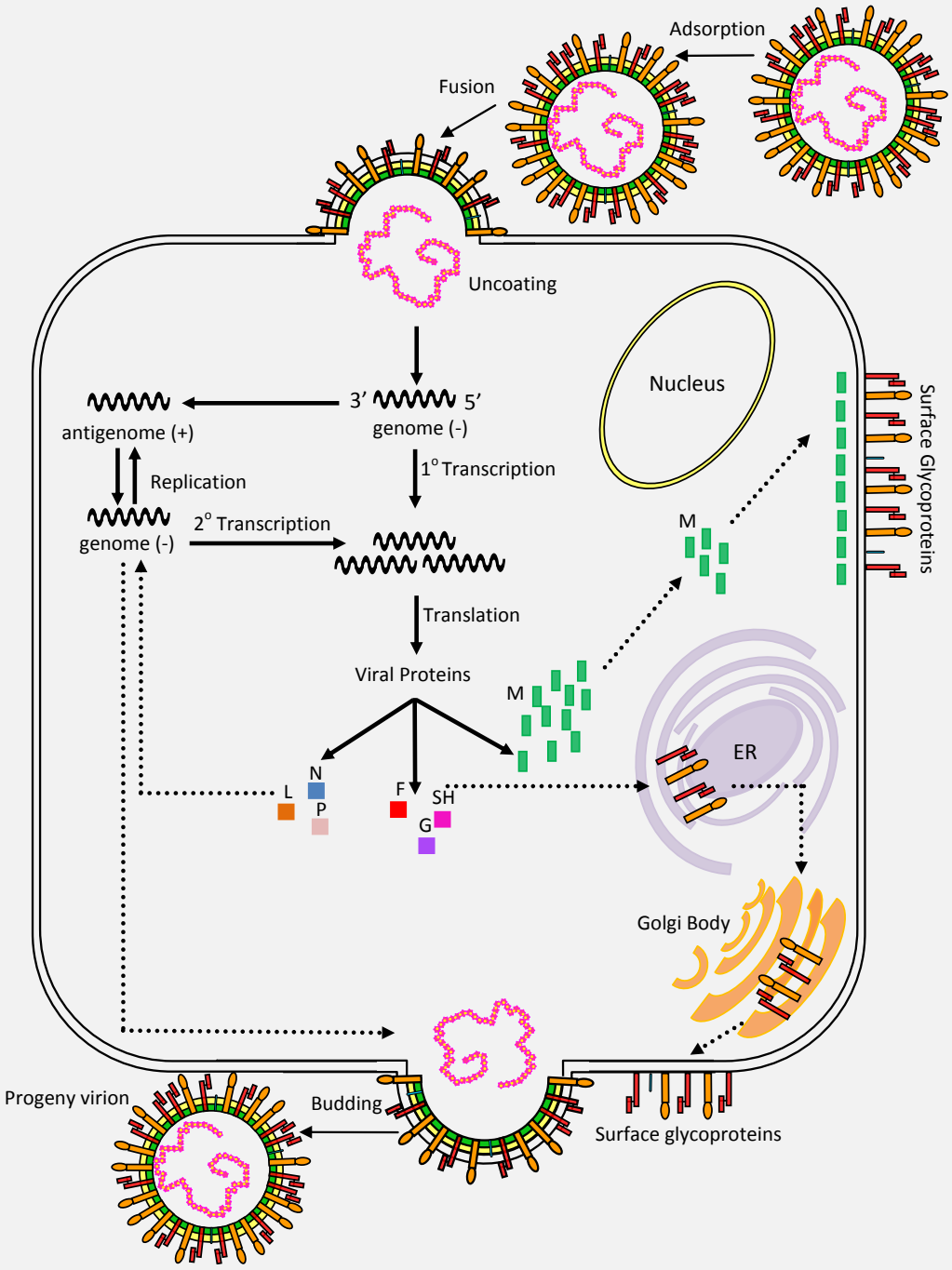


Figure 1.5. Schematic representation of the life cycle of members of the family *Paramyxoviridae*. Adsoption of the virion to the plasma membrane is followed by fusion of the viral envelope with the host cell plasma membrane and delivery of the nucleocapsid into the cytoplasm. Dotted lines denote the post translation intracellular transport of nucleocapsid (N) and matrix (M) proteins to the plasma membrane and the attachment (G), fusion (F) and small hydrophobic (SH) viral glycoproteins from the endoplasmic reticulum (ER) to Golgi body to plasma membrane. Progeny virions are released from the plasma membrane by budding. The idealised illustration is adapted from Lamb and Parks (2007).

1.4.4.2. Viral RNA Synthesis

The consequence of the fusion reaction is that the helical nucleocapsid containing the single-stranded negative sense RNA genome is released into the cytoplasm (Lamb and Parks, 2007). At early times of infection, this genome directs the synthesis of (+) leader and viral messanger RNA (mRNA). After translation of the primary transcripts and accumulation of the viral proteins, the (-) sense genome is replicated to produce a full-length complimentary copy, called the antigenome, which is found only in a form that is assembled with N protein. After synthesis, the antigenomic RNA is used as a template to direct synthesis of genomic RNA. These progeny (-) sense genomes can serve three subsequent functions: as a template for mRNA synthesis in a phase called "secondary transcription", as a template to produce additonal antigenomes, or for incorporation into progeny virions during the budding process (Lamb and Parks, 2007). The viral gene junctions of members of the *Paramyxoviridae t*hat modulate transcription can be divided into three segments: a gene end (GE) region at the 3' end of the upstream gene, the intergenic region (IG) between the two genes that is normally not transcribed, and a gene start (GS) region for the downstream 5' gene. The GE region contains a signal directing the viral RNA polymerase to terminate transcription while reinitiation of transcription is directed by sequences at the downstream GS site. The frequency of reinitiation is not perfect and not every viral RNA polymerase that terminates at a GE remains on the template to reinitiate transcription at the next GS. This imperfect reinitiation leads to a gradient of mRNA abundance that decreases according to distance from the 3' genome end (Lamb and Parks, 2007).

1.4.4.3. Virion Assembly and Release

Our understanding of the replication of HRSV provides a framework to generate hypotheses regarding early virus assembly events in HMPV replication (Derdowski *et al*., 2008). Progeny virions are formed by a budding process from cellular membranes (Harrison *et al*., 2010). Infectious particles will form only after all the structural components of the viruses including viral glycoproteins and viral RNP have assembled at selected sites on infected cell plasma membranes in preparation for particle budding (Harrison *et al*., 2010). Viral M proteins are the organisers of this assembly process. These highly abundant viral proteins bind directly to cellular membranes and occupy a central position that allows interaction both with viral RNP cores and with viral glycoproteins via the cytoplasmic tails. Thus, M proteins are adapters that link together the structural components of virions, driving their assembly (Harrison *et al*., 2010).

1.4.5. Laboratory Diagnosis

1.4.5.1. Cell Culture

The initial report by van den Hoogen *et al*., (2001) identified that HMPV replicated slowly in tertiary monkey kidney (tMK) cells, very poorly in Vero (African green monkey kidney) and human lung adenocarcinoma (A-549) cells and could not be propagated in Madin Darby canine kidney (MDCK) or chicken embryo fibroblasts (CEF) cells (van den Hoogen *et al*., 2001). In a later study, Boivin *et al*., (2002) inoculated respiratory specimens onto the following continuous cell lines: human laryngeal carcinoma (HEp-2), human foreskin fibroblast, Vero, Mink Lung, A-549, human rhabdomyosarcoma (RD), transformed human kidney (293), and human colon adenocarcinoma (HT-29), MDCK and rhesus monkey kidney (LLC-MK2). The virus grew only on LLC-MK2 cells. Peret *et al*. (2002) recovered HMPV in LLC-MK2 but no cytopathic effect was noted in MDCK or human pulmonary mucoepidermoid carcinoma (NCI-H292) cells. Chan *et al*., (2003) inoculated multiple cell lines including LLC-MK2, HEp-2, MCDK, human embryonic lung fibroblast, Buffalo green monkey kidney (BGM) and Vero monolayers with respiratory specimens. A cytopathic effect of focal refractile rounding of cells developed in LLC-MK2 monolayers but was not observed in HEp-2, MCDK, human embryonic lung fibroblast, BGM, and Vero cell monolayers. However, in contrast to these previous studies Chan *et al*., (2003) used RT-PCR in combination with conventional virus isolation to enhance the detection of HMPV in each cell line. With this combination approach, Chan *et al*., (2003) revealed that HEp-2 and MDCK in addition to LLC-MK2 support the growth of HMPV. Furthermore, this study showed the sensitivity of RT-PCR in combination with conventional virus isolation was greater than direct detection of HMPV in NPA by RT-PCR alone. Chan *et al*., (2003) retrospectively examined 25 NPA samples previously positive for HMPV by RT-PCR alone and in combination with conventional virus isolation using HEp-2, LLC-MK2 and MDCK cell monolayers. All first round PCR products derived from direct NPA samples were negative for HMPV indicating nested PCR was required in order to determine the status of the samples. Overall, the sensitivity of direct detection of NPA samples by HMPV RT-PCR was only 8%. In contrast, 88% of those first round PCR products derived from cell cultures were positive for HMPV. Furthermore, Chan *et al*., (2003) identified that HEp-2 cell monolayers were superior to LLC-MK2 and MDCK cell lines for the isolation of HMPV with 88% of HEp-2 cultures positive for HMPV by RT-PCR while HMPV was detected in only 24% of LLC-MK2 cell cultures and 8% of MDCK cell cultures.

To ascertain that cell cultures with HMPV RT-PCR positive results represented the isolation of HMPV, all LLC-MK2, HEp-2, and MDCK cell cultures were passaged to LLC-MK2 cells for prolonged incubation of 28 days. The results showed that all passages from HMPV RT-PCR positive cell cultures showed a characteristic cytopathic effect of focal refractile rounding of cells without syncytia formation that occurred after 10 to 22 days of incubation that progressed slowly to detachment from the cell monolayer (Figure 1.6) (Chan *et al*., 2003). The supernatants of these passages were also positive by HMPV RT-PCR and had visible viral particles on electron microscopic examination (Chan *et al*., 2003). The passages from HMPV RT-PCR negative supernatants did not show positive results by the aforementioned tests (Chan *et al*., 2003). However, these findings do not present an effective argument towards a recommendation to discard LLC-MK2 cells in favour of HEp-2 cells since in 12% of cases HMPV was isolated from LLC-MK2 cells alone (Chan *et al*., 2003). Subsequent studies have demonstrated that human bronchiolar epithelial cells (16HBE140) are superior to LLC-MK2 for the isolation of virus from nasopharyngeal secretions even in the absence of exogenous trypsin (Ingram *et al*., 2006) and more recently, a cell line derived from human hepatoma (HepG2) was shown to support replication of HMPV to high titres (Schildgen *et al*., 2010).

1.4.5.2. Immunofluorescence

The immunofluorescent antibody test is a rapid and inexpensive method that is commonly utilised within routine diagnostic virology laboratories for the rapid detection of respiratory viruses in clinical samples and confirmation of the presence of viruses in cell culture. Development of specific monoclonal antibodies (MAbs) to HMPV is an important advance in the field of rapid direct diagnosis of respiratory tract viral infections (Percivalle *et al*., 2005). The practical difficulties inherent in the routine isolation of HMPV from clinical samples (Reina *et al*., 2007) confirm that virus culture is not suited to timely diagnosis of this important respiratory pathogen in a clinical setting. However, implementation of immunofluorescent staining of shell vial centrifugation cultures (SVCC) with a specific murine monoclonal antibody (MAb-8) to HMPV matrix protein has achieved greater success in the rapid diagnosis of respiratory tract infection caused by this virus (Ingram *et al*., 2006; Landry *et al*., 2005; Reina *et al*., 2007). Indeed, detection of HMPV in A549, HEp-2, and LLC-MK2 SVCC was achieved by MAb-8 staining on day 2 post-inoculation offering a great advantage over conventional culture methods (Landry *et al*., 2005) with LLC-MK2 and HEp-2 displaying 100% and 68.7% sensitivity, respectively (Reina *et al*., 2007).

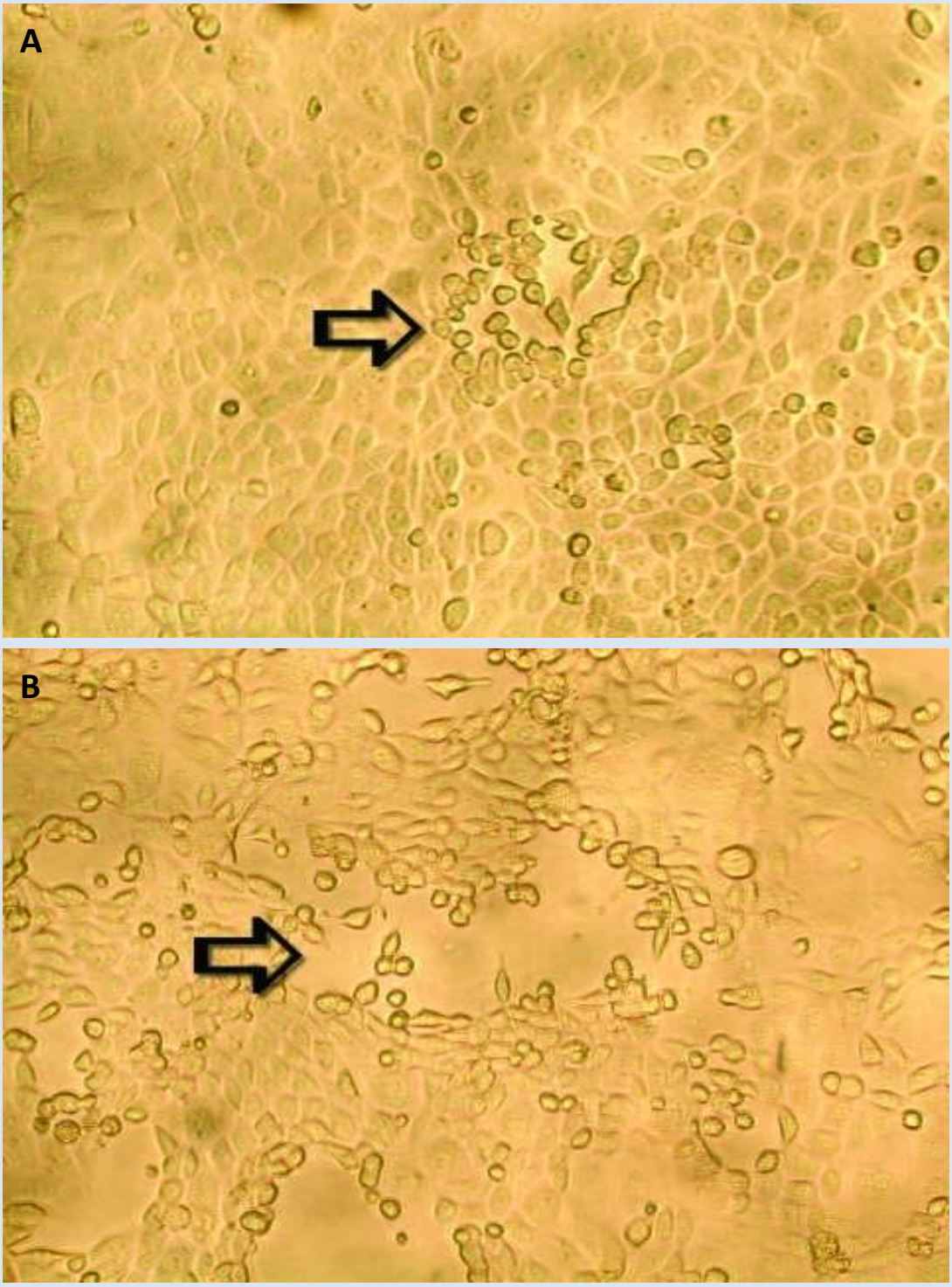


Figure 1.6. Cytopathic effect of human metapneumovirus in rhesus monkey kidney cell monolayers. (A) Early cytopathic effect (CPE) of human metapneumovirus (HMPV) in rhesus monkey kidney (LLC-MK2) cell monolayers. A focus of infected cells that exhibit refractile rounding is indicated by an arrow (100X). (B) Late CPE of HMPV in LLC-MK2 cell monolayers. Infected cells progressed slowly from focal rounding to detachment from cell monolayer is indicated by an arrow (100X) (Chan et al., 2003). Available from URL: http://www.cdc.gov/ncidod/EID/vol9no9/03-0304.htm.

In contrast, the use of MAb-8 in staining clinical samples proved less successful due to non-specific background staining (Landry *et al*., 2005) though the problem of non-specific staining was not limited to the use of MAb-8 (Ebihara *et al*., 2005; Percivalle *et al*., 2005). Indeed, the application of immunofluorescent antibody tests using specific MAbs to the detection of HMPV antigens in nasopharyngeal secretions for the rapid diagnosis of HMPV infection has achieved variable success. In a study comparing IFA with RT-PCR for detection of HMPV in nasal secretions from 48 hospitalised children with respiratory tract infections, Ebihara *et al*., (2005) found IFA results were positive for 11 of the 15 RT-PCR-positive children (sensitivity, 73.3%) and 1 of the 33 RT-PCR-negative children (specificity, 97.0%). Similarly, Percivalle *et al*., (2005) showed sensitivity and specificity of 73.9% and 94.1%, respectively for DFA staining of nasopharyngeal aspirates using RT-PCR as a reference method. However, the choice of MAbs in these early studies was limited and based on the ability of antibodies to stain virus in cell cultures (Fenwick *et al*., 2007). In contrast, later studies reported results comparable to those obtained by RT-PCR (Landry *et al*., 2008; Manoha *et al*., 2008; Vinh *et al*., 2008; Zhang *et al*., 2009) to allow immunofluorescence testing to retain a clinical advantage over PCR as a first-line test (Landry *et al*., 2008). Nevertheless, DFA testing is manual and requires a committed, well-trained, expert staff and results are highly laboratory dependent (Landry *et al*., 2008). This is exemplified in three separate evaluations of a commercially manufactured DFA kit for the detection of HMPV (D^3 DFA Metapneumovirus Identification Kit; Diagnostic Hybrids, Athens, OH, USA). Vinh *et al*., (2008) and Zhang *et al*., (2009) achieved comparable sensitivities of 90% and 95.2% and specificities of 100% and 100%, respectively. However, with a sensitivity of 62.5% and specificity of 99.8%, a marked variation in the performance of the DFA kit was attained in the third evaluation (Aslanzadeh *et al*., 2008).

1.4.5.3. Serology

Serological testing only permits a retrospective diagnosis (Hamelin *et al*., 2004). Since infection is almost universal in childhood, a seroconversion or a $\geq$ 4-fold increase in antibody titres must be demonstrated to confirm recent infection (Hamelin *et al*., 2004). Numerous studies have concentrated efforts towards the serological diagnosis of HMPV infection. Serological tests have been based on the indirect immunofluorescence assays or enzyme-linked immunosorbent assays (ELISA) using HMPV infected tMK (Boivin *et al*., 2002; Ebihara *et al*., 2003; Ebihara *et al*., 2004c; van den Hoogen *et al*., 2001), Vero (Wolf *et al*., 2003; Zhang *et al*., 2008) or LLC-MK2 cells (Falsey *et al*., 2003) or recombinant viral proteins (Leung

et al., 2005; Hamelin *et al*., 2005). In a different study, Falsey *et al*. (2003) developed an ELISA by coating microtitre plates with the lysate of HMPV infected LLC-MK2 cells. In a recent study, Alvarez *et al*. (2004) described the generation of 2 unique antibody reagents for the identification of metapneumoviruses using three different common diagnostic techniques: western blotting, ELISA, and immunohistochemistry (IHC). The first antibody reagent, anti-N protein[10-29] antibody is the first reagent described that detects all members of the genus *Metapneumovirus*. The second antibody reagent, monoclonal antibody 22B3 is the first reagent described that differentiates HMPV and avian metapneumovirus group C from avian metapneumovirus group A and B (Alvarez *et al*., 2004).

1.4.5.4. Reverse-transcription polymerase chain reaction

The difficulties associated with conventional cell culture as a method of detection and the absence of specific diagnostic reagents designed for this purpose led to the need for a reliable, sensitive, and rapid diagnostic method. RT-PCR and real-time RT-PCR are two techniques that have fulfilled this requirement. These techniques provide the ultimate detection system at present. However, as the story of HMPV continues to unfold the associated demands on these techniques continue to grow. The detection of HMPV by RT-PCR in initial studies relied on primers directed toward the L gene (Peiris *et al*., 2003; Stockton *et al*., 2002), F gene (Boivin *et al*., 2002; Esper *et al*., 2003; Peret *et al*., 2002), M gene, (Boivin *et al*., 2002; Peiris *et al*., 2003) and N gene (Freymuth *et al*., 2003; Peret *et al*., 2002). These initial studies demonstrated RT-PCR assays directed at these genes were capable of detecting this novel virus. Indeed, several studies using primers targeting the L gene, (Peret *et al*., 2002; Stockton *et al*., 2002), F gene (Pelletier *et al*., 2002; Peret *et al*., 2002; Boivin *et al*., 2002), and N gene (Peret *et al*., 2002) detected viruses from two distinct genetic lineages designated A and B. Yet, no systematic evaluation was conducted in this intense early period to determine the most appropriate gene target or the limits of sensitivity of such different RT-PCR assays to ensure optimal detection of both the identified genetic lineages of HMPV. However, Cote *et al*. (2003) performed a comparative evaluation of real-time RT-PCR assays that were designed to amplify the N, M, F, P, and L genes in order ascertain the best diagnostic target for the optimal detection of HMPV. In the first evaluation of 20 viral cultures exhibiting a cytopathic effect characteristic of HMPV, the PCR positivity rates were 100, 90, 75, 60, and 55% using a primer and probe set directed at the N, L, M, P, and F genes, respectively. Due to the superior sensitivity of the primers targeting the N gene, a thorough evaluation of this real-time RT-PCR assay was undertaken. In this second

evaluation, 10 NPA specimens from children with bronchiolitis previously positive by the real-time RT-PCR assay targeting the N gene were tested by those real-time RT-PCR assays designed to target the L, M, P and F genes. The positivity rates for the L, M, P, and F genes were 90, 60, 30, and 80%, respectively. This study showed that real-time RT-PCR assays based on N and L gene sequences of HMPV demonstrated the greatest diagnostic potential probably because they targeted more conserved regions of the genome (Cote *et al*., 2003). Mackay *et al*. (2003) identified the diagnostic potential of real-time RT-PCR assay for the detection of this new virus in an earlier study. In this study, 329 NPA samples from patients with respiratory symptoms for which an etiologic agent was not initially detected were tested by a novel conventional RT-PCR assay. The design of the oligonucleotide primer set was based on the N gene sequence of HMPV. Of the 329 samples, 32 (9.7%) were HMPV positive by this assay. To reduce the turn-around time of the conventional RT-PCR assay the existing primer set was combined with a fluorogenic TaqMan oligoprobe. This real-time RT-PCR detected HMPV in an additional 6 out of 62 samples (9.6%) during a comparison of the two diagnostic methods (Mackay *et al*., 2003).

A study by van den Hoogen *et al*., (2004b) showed that the two major genetic lineages of HMPV identified in earlier studies each comprised two sublineages designated A1, A2, B1, and B2. This discovery demanded the invention of a real-time RT-PCR assay with the capacity to detect all genetic sublineages of HMPV described to date. Maertzdorf *et al*. (2004) examined the influence of mismatches in primer and probe sequences described in earlier studies by Mackay *et al*. (2003) and van den Hoogen *et al*. (2003) on the capacity of these primers and probes to detect all four genetic lineages of HMPV. The results obtained indicated that the primer and probe sequences for the N gene (Mackay *et al*., 2003) and L gene (van den Hoogen *et al*., 2003) did amplify target sequences of HMPV belonging to lineage A but not lineage B. Entropy plots of oligonucleotide annealing sites in the N and L genes of HMPV were created with the entropy algorithm available from the BioEdit software programme (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The entropy plots showed mismatches of the primer and probe sequences with the target sequences indicating why the primer and probe sequences described by Mackay *et al*. (2003) and van den Hoogen *et al*. (2003) targeted at the N and L genes, respectively, failed to successfully detect of all four genetic lineages of HMPV (Maertzdorf *et al*., 2004). The primers and probes described by Mackay *et al*. (2003) and van den Hoogen *et al*. (2003) were designed on the basis of limited sequence information primarily available for HMPV belonging to lineage A. Hence,

mismatches between primer and probe sequences and the respective target sequences were most severe for viruses belonging to lineage B (Maertzdorf *et al*., 2004).

The recent identification of a further bipartition of subgroup A2 (Huck *et al*., 2006) reiterates that there is still much to discover about this novel virus. Even RT-PCR and real-time RT-PCR, methods widely used and indeed heavily relied upon due to the improved sensitivity of these techniques present continuous challenges. Studies of the heterogeneity of HMPV indicate the importance of optimisation of RT-PCR and real-time RT-PCR protocols in order to ensure optimal detection of HMPV. It is clear that there is no room for complacency. As a solution to these diagnostic difficulties, Chan *et al*. (2003) present an algorithm for the detection of HMPV that incorporates RT-PCR in combination with conventional virus isolation to enhance detect HMPV. This combination approach may enable genetic lineages of HMPV that remain undiscovered at present to be identified.

1.4.6. Clinical importance

Acute respiratory tract infection (ARTI) is a leading cause of morbidity and mortality with a global burden of disease (GBD) estimated at 94 037 000 disability adjusted life years (DALYs) and 3.9 million deaths (WHO, 2002). Acute respiratory tract infections are the most common illnesses regardless of age or gender (Monto, 2002). The burden of these infections is not only in the loss of lives. Acute respiratory tract infections are a substantial drain on meagre health resources. The viruses primarily associated with ARTI in children and adults include influenza A and B viruses, HPIV types 1, 2, and 3, HRSV, adenovirus, and human rhinovirus (HRV) (Mahony, 2008). Nevertheless, the aetiology of a substantial number of these infections remains unknown (Davies *et al*., 1996; Nokso-Koivisto *et al*., 2002; Ruiz *et al*., 1999; Wright *et al*., 1989). Our assumption has been that the gap is attributable to a combination of the insufficient sensitivity of available diagnostic techniques and the existence of undiscovered respiratory pathogens. Both of these assumptions turn out to be correct (McIntosh and McAdam, 2004). This gap in sensitivity was considerably narrowed following the introduction of PCR to the diagnosis of viruses. In contrast, gaps associated with undiscovered respiratory pathogens remained largely unfilled (McIntosh and McAdam, 2004). After the discovery of human coronavirus (HCoV) 229E and HCoV OC-43 in the 1960s, there was hope that other members of the family *Coronaviridae* would account for some of the undiscovered respiratory pathogens (McIntosh and McAdam, 2004). Yet, until the abrupt arrival of SARS-CoV, these viruses received relatively little attention as human pathogens

(Arden *et al*., 2005). However, the discovery of HMPV and the novel HCoV strains NL63 and HKU1, represent a significant advance in the investigation of human respiratory tract disease (Kahn, 2006).

The identification of HMPV in children suffering from respiratory tract illness in the Netherlands in 2001 (van den Hoogen *et al*., 2001) sparked an intense flurry of international activity to identify this new virus as the aetiology of respiratory tract infections previously relegated to the "undiagnosed" category (McIntosh and McAdam, 2004) (Table 1.4). HMPV has rapidly emerged as a seemingly important cause of both upper and lower respiratory tract infections in otherwise healthy children, the elderly and immunocompromised patients but is a position on the list of respiratory pathogens justified? In order to establish with a high degree of certainty that HMPV causes ARTI, it is necessary to evaluate the evidence proposed in studies conducted to date. However, it is not possible to consider the information on its own merit since any evidence that exists to link HMPV to ARTI is purely circumstantial. It is therefore necessary to consider this evidence with reference to Koch's postulates in order to prove to sceptics that a causal link between HMPV and ARTI does indeed exist.

Table 1.4. Detection of human metapneumovirus in patients in all age groups with respiratory tract infection

Location	Period of study	Population	No. patients tested	No. positive patients	Peak Age	Peak Period	Reference
Canada	Dec 01-Apr 02	All ages, RTI	862	20 (2.3%)	35% <5yrs	Feb 02	Boivin *et al*., (2002)
Hong Kong	Aug 01 – Mar 02	≤18 yrs, ARTI	587	32 (5.5%)	3 – 72 months	spring-summer	Peiris *et al*., (2003)
The Netherlands	Sept 00 – Feb 02	All ages, RTI	681	47 (7%)	4 – 6 months	Dec – Jan 02	van den Hoogen *et al*., (2003)
Germany	Jan 02 – May 02	<2 yrs, RTI	63	11 (17.5%)	<2 years	Jan – Apr 02	Viazov *et al*., (2003)
Canada	Dec 01 – Apr 02	≤3 yrs, ARTI	208	12 (5.8%)	3 – 5 months	Mar – Apr 02	Boivin *et al*., (2003)
Italy	Jan 00 – May 02 2000 2001 2002	1– 24 months, ARTI	 19 41 30	 7 (37%) 3 (7%) 13 (43%)	1– 24 months	Jan – May	Maggi *et al*., (2003)
USA	Oct 01 – Feb 02	<5 yrs, RTI	296	19 (6.4%)	2.5 – 58.8 months	Jan – Feb 02	Esper *et al*., (2003)
France	Nov 01 – Feb 02	Children, ARTI	337	19 (6.6%)	3 months – 12 yrs	Dec – Jan 02	Freymuth *et al*., (2003)
Canada	Oct 01 – Apr 02	All ages, ARTI	445	66 (14.8%)	2 months – 93 yrs	Feb – Mar 02	Bastien *et al*., (2003)
USA	Aug 00 – Sept 01	<5, ARTI	641	26 (3.9%)	6 – 24 months	Jan – Apr 01	Mullins *et al*., (2004)

1.4.6.1. Koch's postulates

Koch's postulates were formulated in 1886 by Robert Koch as guidelines to establish a standard for identifying the specific causation of an infectious disease (Falkow, 2004; Walker *et al*., 2006). However, the fundamental limitations of Koch's postulates are no more apparent than when applied to obligate parasites. Indeed, since viruses propagate by usurping cellular machinery so propagation in pure culture is simply not possible (Fredericks and Relman, 1996). A notable example is herpes simplex virus. This virus will not grow in cell-free culture but is unequivocally pathogenic (Fredericks and Relman, 1996). Similarly, HIV exhibits a host range that is restricted to humans, thereby making impossible or unethical the fulfilment of the third postulate (Fredericks and Relman, 1996) (Table 1.5).

Table 1.5. Koch's original postulates.

The parasite occurs in every case of the disease in question and under circumstances can account for the pathological changes and clinical course of the disease.
The parasite occurs in no other disease as a fortuitous and non-pathogenic parasite.
After being fully isolated from the body and repeatedly grown in pure culture, the parasite can induce the disease anew.

Adapted from Fredericks and Relman (1996).

In recognition of the weight of evidence supporting the notion that viruses cause specific diseases and the inability of Koch's postulates to incorporate this evidence, the original postulates (Fredericks and Relman, 1996) were modified by Thomas Rivers in 1937 to establish a causal relationship between a virus and a disease (Table 1.6) (Osterhaus *et al*., 2004). If Koch's postulates, as modified by Rivers for virus diseases (Osterhaus *et al*., 2004), are considered in relation to evidence of disease causation by HMPV then fulfilment of the first three criteria, namely the isolation of the virus from diseased hosts, cultivation in host cells and proof of filterability, was achieved early in the discovery of HMPV.

Table 1.6. Koch's postulates as modified by Thomas Rivers (1937).

1.	Isolation of the virus from diseased hosts
2.	Cultivation in host cells
3.	Proof of filterability
4.	Production of a comparable diseases in the original host species or a related one
5.	Re-isolation of the virus
6.	Detection of a specific immunoresponse to the virus

Adapted from Osterhaus *et al*., (2004).

1.4.6.2. Isolation of the virus from diseased hosts

The first step to establish a causal link between a virus and a disease is to isolate the virus from the diseased host. However, the virus must occur in the sick individual not as an incidental or accidental finding but as the cause of the disease under investigation (Rivers, 1937). If the initial study conducted by van den Hoogen *et al*., (2001) is considered HMPV was isolated from nasopharyngeal aspirates taken from 28 epidemiologically unrelated children in the Netherlands suffering from RTI during a period of 20 years. All NPA samples were previously tested as part of routine investigations for the presence of HPIV types 1–3, HRSV and influenza virus types A and B by DFA and virus isolation. Samples showing a CPE after 2 or 3 passages that were negative by DFA were tested by IFA using virus-specific antibodies against influenza virus types A, B and C, HRSV, HPIV types 1–4, MeV, MuV, SeV, SV-5, and NDV. However, while a CPE was identified in cell cultures, routine investigations failed to identify an aetiological agent. Therefore, it was reasonable to suggest even at this very early stage in the discovery of HMPV that a tenable link was visible between the virus and a disease i.e. ARTI. Later studies confirmed that HMPV was associated with RTI in patients with respiratory illnesses not caused by other respiratory viruses (Table 1.7). It is interesting to note the increased detection rates of HMPV within retrospective studies compared to prospective studies, an observation that is consistent with a degree of selection bias (Principi *et al*., 2006) toward patients with respiratory tract disease without an aetiological diagnosis. However, it is essential not to overlook the importance of retrospective studies since these studies not only show that HMPV is associated with respiratory tract infection in symptomatic individuals but that this virus is frequently associated with respiratory tract disease.

A further point worthy of consideration is the method used to detect HMPV. Several of these studies use RT-PCR or real-time RT-PCR for the detection of HMPV. However, the application of these highly sensitive nucleic acid amplification techniques to the diagnosis of infectious diseases has raised concerns about the frequency of RNA detection in non-symptomatic patients and the persistence of nucleic acid and therefore positivity after recovery from illness (Hayden, 2006). It is known that respiratory viruses can persist for prolonged periods in the nasopharyngeal tract without symptoms (Larcher *et al*., 2005). Due to the possible long-term persistence of nucleic acids, an increasing number of studies have used a combination approach based on cell culture and nucleic acid amplification techniques to detect active RTI with HMPV (Williams *et al*., 2004; Larcher *et al*., 2005). In this way, it is possible to provide evidence for the role of HMPV as a new respiratory pathogen and

convince sceptics that this newly identified virus is capable of causing disease. Nevertheless, in the quest to streamline routine diagnostic services many laboratories have replaced tissue culture in favour of nucleic acid amplification techniques. Hence, it is increasingly important to establish the appropriate interpretation of a positive result especially when attempting to provide an unambiguous establishment that a new pathogen is central to a disease process (Gao and Moore, 1996). Indeed, Falsey *et al*., (2006) evaluated nasal secretions from adults with and without respiratory illness by RT-PCR to determine if rates of detectable RNA were significantly higher among ill subjects compared to controls. The virus was detected in 5 of 146 (3.4%) symptomatic adults while 0 of 158 control subjects tested positive for HMPV between January and April 2004, a period during which HMPV activity was expected based on previous epidemiological studies (Falsey *et al*., 2006). Whilst data on asymptomatic carriage of HMPV is currently limited due to the relatively recent discovery of HMPV, these findings confirm those of other studies that asymptomatic carriage of HMPV is an uncommon event (Boivin *et al*., 2003; Bruno *et al*., 2009; Rohde *et al*., 2005; Williams *et al*., 2004; van den Hoogen *et al*., 2004) even during periods of peak activity. This strengthens the causal link between the detection of HMPV and RTI (Falsey *et al*. 2006). Nevertheless, it appears that mild infection characterised by a serological response is relatively common (Walsh *et al*., 2008) particularly in young healthy adults. Indeed, 9.5% of healthy adults <40, 1.4% of healthy adults ≥ 65 years and 1.5% of high-risk adults without respiratory symptoms had evidence of HMPV infection based on serologic responses during the 1999–2001 winter seasons (Falsey *et al*., 2003). In a further study, evidence of asymptomatic infection with HMPV was identified in 71% of healthy adults aged 19-40 years, 44% of healthy adults ≥ 65 years, and 39% of high-risk adults by serological testing (Walsh *et al*., 2008). It is perhaps not surprising that asymptomatic infection with HMPV was most evident in young healthy adults since contact with children was greatest within this cohort (Falsey *et al*., 2003; Walsh *et al*., 2008). These findings suggest that asymptomatic carriage of HMPV might provide a neglected source of viral transmission in the community (Bruno *et al*., 2009).

1.4.6.3. Cultivation in host cells

Despite the difficulties associated with the isolation of HMPV by cell culture, successful propagation of this virus from a variety of respiratory specimens in a limited range of permissive cells is achievable. HMPV replicates efficiently in LLC-MK2 and Vero cell lines (Deffrasnes *et al*., 2005). Subsequent studies have demonstrated that 16HBE140 are superior to LLC-MK2 for the isolation of virus from nasopharyngeal secretions even in the

absence of exogenous trypsin (Ingram *et al*., 2006) while HepG2 was shown to support replication of HMPV to high titres (Schildgen *et al*., 2010).

1.4.6.4. Proof of filterability

HMPV was concentrated from infected tMK-cell supernatants by ultracentrifugation on a 20–60% sucrose gradient (van den Hoogen *et al*., 2001). Gradient fractions were inspected for the presence of virus-like particles by negative contrast electron microscopy and revealed the presence of pleomorphic particles in the range of 150-600 nm, with short envelope projections in the range of 13-17 nm (van den Hoogen *et al*., 2001) suggestive of a paramyxovirus. Consistent with the properties of several members of the *Paramyxoviridae* nucleocapsids were rarely observed (van den Hoogen *et al*., 2001). These combined virological data indicated that this earlier undiscovered virus was a member of the family *Paramyxoviridae* (van den Hoogen *et al*., 2001).

1.4.6.5. Production of a comparable diseases in the original host species or a related one

In order to determine whether HMPV is a primary human pathogen or an avian pathogen that can also infect humans experimental infection of birds and cynomolgus macaques (*Macaca fascicularis*) was performed (van den Hoogen *et al*., 2001). The conjunctivae and respiratory tracts of four juvenile turkeys, four juvenile chickens, and four juvenile cynomolgus macaques were inoculated with 50,000 $TCID_{50}$ (50% tissue culture infectious dose) of HMPV (van den Hoogen *et al*., 2001). None of the birds showed clinical signs or virus replication as determined by RT-PCR using RNA isolated from throat and cloacal swabs (van den Hoogen *et al*., 2001) although the cross-species pathogenicity of HMPV was later demonstrated in turkey poults (Velayudhan *et al*., 2006). In contrast, the virus replicated efficiently in the respiratory tract of all four monkeys as shown by RT-PCR of RNA isolated from throat swabs and 2 monkeys presented with mild upper respiratory tract signs that upon histological analysis proved to be associated with suppurative rhinitis (van den Hoogen *et al*., 2001).

Subsequent experimental infection of cynomolgus macaques established that viral replication was restricted to the respiratory tract and associated with minimal to mild, multi-focal erosive and inflammatory changes in conducting airways (Kuiken *et al*., 2004) and so substantiated that HMPV is a human respiratory pathogen (Kuiken *et al*., 2004).

Studies to identify other non-human primate models that efficiently support the replication of HMPV in the respiratory tract demonstrated HMPV sero-negative African green monkeys support replication of HMPV efficiently and produced high levels of neutralising antibody while only minimal virus replication was observed in rhesus monkeys (*Macaca mulatta*) (MacPhail *et al*., 2004). However, it also appears that the chimpanzee (*Pan troglodytes*) infected with HMPV represents the only "real" infection model (Schlidgen *et al*., 2007) following the findings that 19 of 31 (61%) captive chimpanzees were seropositive for HMPV while the remaining 12 animals (39%) were seronegative (Skiadopoulos *et al*., 2004). These surprising data indicate that chimpanzees are susceptible to natural infection with HMPV thus leading to the assumption that there is no species barrier between humans and chimpanzees regarding HMPV (Schlidgen *et al*., 2007). Indeed, a HMPV was the likely causative agent associated with a fatal respiratory disease outbreak in habituated wild chimpanzees (Kaur *et al*., 2008; Köndgen *et al*., 2008) and wild mountain gorillas (*Gorilla beringei beringe*) (Palacios *et al*., 2011).

1.4.6.6. Re-isolation of the virus

Six cynomolgus macaques were infected with 5.0 X 10^4 $TCID_{50}$ of NL/1/100, the A1 prototype strain of HMPV (Kuiken *et al*., 2004). Clinical signs in HMPV-infected macaques were limited to rhinorrhoea, and corresponded with a suppurative rhinitis at pathological examination (Kuiken *et al*., 2004). Pharyngeal swabs were collected daily from 2 days post infection (dpi) until euthanisation or 10 dpi (Kuiken *et al*., 2004). Animals were euthanised at 5 (*n* = 2) or 9 (*n* = 2) dpi, or monitored until 14 dpi (*n* = 2) (Kuiken *et al*., 2004). The virus was detected in pharyngeal swabs of one or more animals by RT-PCR (Kuiken *et al*., 2004). After an incubation period of 2 days at most, excretion of HMPV increased rapidly to a peak of 1.3 X 10^6 $TCID_{50}$/ml at 4 dpi, and then decreased gradually to zero at 10 dpi (Kuiken *et al*., 2004). The results of RT-PCR were confirmed by virus isolation (Kuiken *et al*., 2004). HMPV was re-isolated from pharyngeal swabs collected at the peak of virus excretion of all six macaques (Kuiken *et al*., 2004).

1.4.6.7. Detection of a specific immunoresponse to the virus

To determine the prevalence of HMPV within the human population, van den Hoogen *et al*. (2001) tested serum samples collected from different age groups. This analysis revealed that 25% of children aged between 6 and 12 months in the Netherlands had antibodies to the virus and by 5 years of age virtually all children were seropositive (van den Hoogen *et al*., 2001). Furthermore, analysis of archived serum samples collected in 1958 from humans aged 8 to 99 years revealed a seroprevalence rate of 100%, indicating that HMPV has been circulating within the human population for at least 50 years (van den Hoogen *et al* 2001).

1.4.7. Clinical manifestations of human metapneumovirus infection

Preliminary data presented by van den Hoogen *et al*. (2001) indicated that among the 28 children infected with HMPV the spectrum of clinical disease was largely similar to that associated with HRSV, ranging from mild upper respiratory tract disease to severe bronchiolitis and pneumonia, often accompanied by high fever, myalgia, and vomiting. Furthermore, some of the children required hospitalisation and mechanical ventilation. Subsequent studies have expanded this initial spectrum of disease associated with HMPV infection to include all age groups as well as risk factors for HMPV infection (Al-Sonboli *et al*., 2006; Boivin *et al*., 2002; Esper *et al*., 2004; Falsey *et al*., 2003; Morrow *et al*., 2006; Paget *et al*., 2011; Robinson *et al*., 2005; Stockton *et al*., 2002; van den Hoogen *et al*., 2003; von Linstow *et al*., 2008; Walsh *et al*., 2008; Wilkesmann *et al*., 2006; Williams *et al*., 2004). Like other common human respiratory viruses, HMPV is associated with upper respiratory tract infection (URTI) (Kahn *et al*., 2006) as well as lower respiratory tract infection (LTRI) but there are also indications that the virus may have a predilection for the central nervous system (Arnold *et al*., 2009; Schildgen *et al*., 2005b). A number of factors are associated with increased risk of acquiring HMPV infection. These include a history of premature birth, underlying heart or lung disease, gastrointestinal reflux disease or aspiration, compromised immune system, exposure to household tobacco smoke or other indoor air pollution, older siblings, and birth in the spring (Al-Sonboli *et al*., 2006; Chen *et al*., 2010; McAdam *et al*., 2004; Mullins *et al*., 2004; Pelletier *et al*., 2002; Robinson *et al*., 2005; von Linstow *et al*., 2008; Wilkesmann *et al*., 2006; Williams *et al*., 2010). Furthermore, several studies have reported a trend toward male predominance in children with HMPV infection (Chan *et al*., 2007; Esper *et al*., 2004; Ljubin-Sternak *et al*., 2008; McAdam *et al*., 2004; Morrow *et al*., 2006; Peiris *et al*., 2003; Samransamruajkit *et al*., 2006; Williams *et al*., 2004) although this trend is not universal (Boivin *et al*., 2003; Pizzorno *et al*., 2010).

Hospitalised children with ARTI attributable to HMPV now represent the most extensively studied patient group (Hamelin *et al*., 2004). Estimates of the incidence rate of HMPV among children hospitalised with ARTI range from 2.5% to ≈ 15% (Table 1.7) although higher incidence rates have been observed (Caracciolo *et al*., 2008; Døllner *et al*., 2004; Maggi *et al*., 2003) that reflect the variable epidemiologic and molecular characteristics of virus. The virus is recognised as a leading cause of hospitalisation for ARTI in children <5 years of age (Williams *et al*., 2010). However, children <2 years of age are at greatest risk of hospitalisation (Beneri *et al*., 2009; Boivin e*t al*., 2003; McAdam *et al*., 2004; Mullins *et al*., 2004; Nicholson *et al*., 2006; Williams *et al*., 2010). Nevertheless, it is evident that children with HMPV infection are significantly older than children with HRSV infection (Baer *et al*., 2008; Camps *et al*., 2008; Morrow *et al*., 2006; Mullins *et al*., 2004; Peiris *et al*., 2003; Wolf *et al*., 2006). This is attributed to longer-lasting maternal immunity to HMPV compared to HRSV or perhaps the pathogenesis of HMPV disease favours older children (Mullins *et al*., 2004).

Most studies have focused almost exclusively on the aetiologic role of HMPV among hospitalised children with RTI in comparison to HRSV (Ali *et al.,* 2010; Al-Sonboli *et al*., 2006; Boivin *et al.,* 2004; Caracciolo *et al.,* 2008; Foulongne *et al*., 2006; Morrow *et al.,* 2006; Mullins *et al*., 2004; Oliveira *et al.,* 2009; Viazov *et al.,*2003; Wolf *et al.,* 2006), the most important cause of serious respiratory tract infection in infants and young children (Parrott *et al*., 1973). While risk factors for HMPV and HRSV hospitalisation appear to be similar (Robinson *et al*., 2005) controversy remains regarding the disease severity of HMPV compared with HRSV. Indeed, some studies reveal that infection with HRSV is associated with a more severe respiratory picture as evidenced by longer duration of symptoms (Viazov *et al*., 2003) or hospitalisation (Chan *et al*., 2007; Marguet *et al*., 2009) as well as more frequent admission to the intensive care unit (Boivin *et al*., 2003; Calvo *et al*., 2010), requirement for supplemental oxygen (Chan *et al*., 2007; van den Hoogen *et al*., 2003) or respiratory support (von Linstow *et al*., 2004). However, a growing number of reports exist that show that infection attributable to HMPV displays a clinical spectrum and disease severity that is indistinguishable from HRSV infection in hospitalised patients (Baer *et al*., 2008; Mullins *et al*., 2004; Paget *et al*., 2011; Rigal *et al*., 2010; Sung *et al*., 2011; Viazov *et al*., 2003; Wilkesmann *et al*., 2006; Wolf *et al*., 2010). This underscores the clinical importance of HMPV which is now recognised as the second most frequent infectious aetiological agent in children hospitalised with RTI after HRSV (Foulongne *et al*., 2006; García-García *et al*., 2006a; Ginocchio *et al*., 2008; Madhi *et al*., 2007a; Noyola *et al*., 2005; Sarasini *et al*., 2006).

Table 1.7. Incidence of human metapneumovirus among children hospitalised with acute respiratory tract infection.

Period of study	Population	No. Patients Tested	No. Positive Patients (%)	Reference
Aug 01 – Mar 02	≤18 yrs	587	32 (5.5)	Peiris *et al*., (2003)
Dec 01 – Apr 02	≤3 yrs	208	12 (5.8)	Boivin *et al*., (2003)
Jan 00 – May 02 2000 2001 2002	1– 24 months	 19 41 30	 7 (37) 3 (7) 13 (43)	Maggi *et al*., (2003)
Nov 01 – Feb 02	Children	337	19 (6.6)	Freymuth *et al*., (2003)
Jul 01 – Nov 01	Children	75	4 (5)	Jennings *et al*., (2004)
Nov 02 – Apr 03	1 – 115	236	50 (21)	Døllner *et al*., (2004)
Aug 00 – Sept 01	<5 yrs	641	26 (3.9)	Mullins *et al*., (2004)
Nov 02 – Mar 03	<15 yrs	1019	42 (4.1)	Bosis *et al*., (2005)
Oct 02 – June 03 July 03 – June 04	<3 yrs	131 192	8 (3.2) 26 (8.4)	Noyola *et al*., (2005)
Oct 02 – May 03	≤2 yrs	601	66 (11)	Al-Sonboli *et al*., (2006)
Dec 03 – Feb 05	<15 yrs	381	28 (7.3)	Chung *et al*., (2006)
Oct 00 – Jun 03	<2 yrs	748	69 (9)	García-García *et al*.,(2006a)
Oct 00 – June 05	<2 yrs	1322	10 (7.6)	García-García *et al*., (2006b)
Dec 03 – May 04	Children	306	40 (13.1)	Sarasini *et al*., (2006)
Nov 03 – May 04 Nov 04 – May 05	<5 yrs	279 499	22 (7.8) 32 (6.4)	Bonroy *et al*., (2007)
Sept 03 – Apr 05	<15 yrs	726	33 (4.5)	Chan *et al*., (2007)
Dec 02 – Apr 03 May 03 – Apr 04	<3 yrs	375 521	38 (10.1) 17 (3.3)	Manoha *et al*., (2007)
Oct 00 – Oct 07	<2 yrs	1612	10 (6.8)	Aberle *et al*., (2008)
Oct 04 – Sept 06	<24 months	322	46 (14.3)	Canducci *et al*., (2008)
Oct 05 – Apr 06 Oct 06 – Apr 07	<5 yrs	154 193	39 (25.3) 9 (4.7)	Caracciolo *et al*., (2008)
Jan 05 – Apr 07	<16 yrs	1214	10 (8.4)	Chung *et al*., (2008)
Dec 03 – May 04	<5 yrs	326	8 (2.5)	Kaplan *et al*., (2008)
Dec 05 – Mar 06	≤5 yrs	402	33 (8.2)	Ljubin-Sternak *et al*., (2008)
July 04 – June 07	≤3 yrs	796	90 (11.3)	Cilla *et al*., (2009)
1987 – 2008	<2 yrs	3576	20 (5.6)	Aberle *et al*., (2010)
Jan 07 – Mar 07	<5 yrs	743	44 (6)	Ali *et al*., (2010)
Oct 01 – Sept 03	<5 yrs	1104	42 (3.8)	Willams *et al*., (2010)
Nov 04 – Jan 08	<15 yrs	309	21 (7)	Do *et al*., (2011)
2004 – 2008	≤3 yrs	240	20 (8.3)	Zappa *et al*., (2011)
Dec 08 – Dec 09	≤15 yrs	575	29 (5)	Zuccotti *et al*., (2011)

1.4.7.1. Upper Respiratory Tract Infections

The role of HMPV in the aetiology of URTI in otherwise healthy children was the focus of a retrospective study based over a 20-year period (Williams *et al*., 2006b). Children with URTI associated with HMPV presented with typical symptoms including fever, coryza, cough, hoarseness, otalgia, rhinitis, conjunctivitis, pharyngitis, and abnormal tympanic membrane (Williams *et al*., 2006b). These clinical symptoms were widely reported in addition to nasal congestion, rhinorrhoea, tachypnoea, dyspnoea, stridor, diarrhoea, febrile seizures, rash and feeding difficulties (Beneri *et al*., 2009; Boivin *et al*., 2002; Esper *et al*., 2003; Pelletier *et al*., 2002; Peiris *et al*., 2003; van den Hoogen *et al*., 2003; Sloots *et al*., 2006).

The crucial role of respiratory viruses in the aetiology and pathogenesis of acute otitis media (AOM) is well established (Bakaletz, 2010). Therefore, it is no surprise that HMPV is associated with AOM in children (Heikkinen *et al*., 2008; Schildgen *et al*., 2005a; Suzuki *et al*., 2005; Williams *et al*., 2006a, b). Indeed, the high rate of AOM as a complication of HMPV-associated RTI (Heikkinen *et al*., 2008; Williams *et al*., 2006b) suggests that HMPV is one of the major viruses predisposing children to AOM (Heikkinen *et al*., 2008). Moreover, the presence of HMPV in 8/144 (6%) of nasal wash specimens supports the role of HMPV in antecedent URTI that predisposes to the development of AOM (Williams *et al*., 2006a). A definitive role for HMPV in middle ear pathogenesis could not be established based on the finding of HMPV in 1/144 middle ear fluid (MEF) samples collected from children with AOM. Nevertheless, no bacterial pathogen was isolated from 2/8 (25%) children with HMPV infection suggesting HMPV may be associated with AOM as a sole pathogen (Williams *et al*., 2006a).

1.4.7.2. Lower Respiratory Tract Infections

A longitudinal study conducted over a period of 25 years provided the first detailed insight of the aetiologic role of HMPV in LRTI among otherwise healthy children (Williams *et al*., 2004). HMPV is a frequent cause of hospitalisations among children with LRTI (Table 1.8) and is second only to HRSV as the viral aetiology of LRTI in hospitalised children in several studies (Chun *et al*., 2009; Escobar *et al*., 2009; Madhi *et al*., 2007a; Sung *et al*., 2011; Zucotti *et al*., 2011). Clinical symptoms among children with HMPV infection of the lower respiratory tract are characterised primarily by preceding URTI, cough, coryza, fever, cyanosis, and dyspnoea and to a lesser extent irritability, anorexia, diarrhoea, and vomiting while signs of HMPV LRTI include tachypnoea, wheezing, retractions, rhonchi and rales (Chen *et al*., 2007; Døllner *et*

al., 2004; Estrada *et al*., 2007; Morrow *et al*., 2006; Nascimento-Carvalho *et al*., 2011; Samransamruajkit *et al*., 2006; Thomazelli *et al*., 2007; Williams *et al*., 2004; Wolf *et al*., 2006; Zucotti *et al*., 2011). The most frequent diagnoses in hospitalised children with LRTI attributable to HMPV are bronchitis, bronchiolitis, croup, pneumonia, exacerbation of bronchopulmonary dysplasia (BPD), and exacerbation of asthma (Boivin *et al*., 2003; Chen *et al*., 2010; Legrand *et al*., 2011; Ljubin-Sternak *et al*., 2008; McAdam *et al*., 2004; Mullins *et al*., 2004; Oliveira *et al*., 2009; Samransamruajkit *et al*., 2006; Williams *et al*., 2004; Williams *et al*., 2010).

Table 1.8. Incidence of human metapneumovirus among children hospitalised with acute lower respiratory tract infection.

Period of study	Population	No. Patients Tested	No. Positive Patients (%)		Reference
Apr 02 – May 02 Apr 03 – May 03	<5 yrs	111 106	27 0	(24) (0)	Serafino *et al*., (2004)
Sept 00 – Aug 05	≤5 yrs	515	24	4.7)	Choi *et al*., (2006)
Mar 01 – Sept 03	Children	220	12	(5.4)	Samransamruajkit *et al*., (2006)
Nov 01 – Oct 02	<5 yrs	516	68	(13)	Wolf *et al*., (2006)
Jan 03 – Dec 03	<5 yrs	336	60	(17.8)	Thomazelli *et al*., (2008)
Jan 03 – Dec 04	<2 yrs	545	56	(10.2)	Escobar *et al*., (2009)
Jan 03 – Dec 06	≤5 yrs	1670	191	(11.4)	Oliveira *et al*., (2009)
Apr 06 – Mar 08	Children	878	227	(25.9)	Chen *et al*., (2010)
May – Nov 2006 2007	<2 yrs	 208 9	 17 1	 (8.2) (11.1)	Pizzorno *et al*., (2010)
Oct 05 – Sept 07	<3 yrs	440	66	(15)	Singleton *et al*., (2010)
Nov 02 – Mar 03 Nov 03 – Mar 04 Nov 04 – Mar 05	Children	1040 263 1112	59 17 52	(5.6) (6.4) (4.7)	Legrand *et al*., (2011)
Aug 08 – Aug 09	Children	169	13	(7.7)	Sánchez-Yebra *et al*., (2011)
Apr 07 – Dec 07	<36 months	48	13	(27.1)	Sung *et al*., (2011)

Chest radiography of the lungs of hospitalised children with HMPV LRTI reveal diffuse perihilar infiltrates (Figure 1.7), hyperinflation, peribronchial cuffing, bronchial wall thickening, focal consolidation and atelectasis (Esper *et al*., 2004; Morrow *et al*., 2006; Samransamruajkit *et al*., 2006; Williams *et al.,* 2004; Wolf *et al.,* 2006; van den Hoogen *et al*., 2003) as the most common abnormalities. Some evidence exists that atelectasis is more common among children with HMPV infection in comparison to children with HRSV or influenza virus type A infections (Wolf *et al.,* 2006), which may provide an important adjunct in the differential diagnosis of RTI in children.

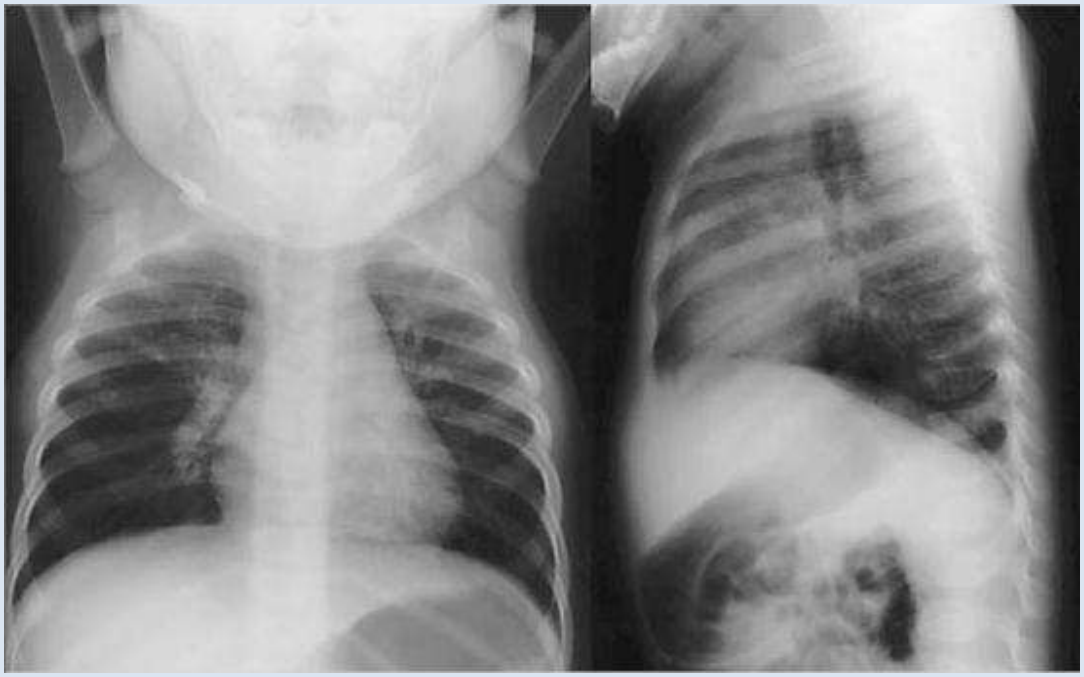


Figure 1.7. Chest radiograph obtained in a six-month-old Infant with human metapneumovirus bronchiolitis showing hyperinflation and diffuse perihilar infiltrates.
Image taken from Williams *et al*., (2004).

While it is clear that HMPV causes a similar range of clinical presentations to HSRV (Boivin *et al*., 2003; Morrow *et al*., 2006; Mullins *et al*., 2004; Sung *et al*., 2011; Wolf *et al*., 2006) debate exists regarding the primary manifestation of HMPV infection in hospitalised children. Evidence exists that bronchiolitis is the main syndrome associated with HMPV infection in hospitalised children (Bovin *et al*., 2003; Døllner *et al*., 2004; Williams *et al*., 2004) while a diagnosis of pneumonia is made less frequently (Morrow *et al*., 2006). In contrast, other studies show that pneumonia is the primary manifestation of HMPV infection in this patient group (Camps *et al*., 2008; Samransamruajkit *et al*., 2006; Wolf *et al*., 2006). These variable results may reflect the capacity of the virus to cause a spectrum of lower respiratory illness with a tendency toward severe infection (Camps *et al*., 2008).

1.4.7.3. Wheezing and Asthma

A causative role of respiratory viruses in the initiation and progression of asthma remains a controversial issue (Kahn, 2006). While increasing evidence favours an association between HMPV infection and asthma exacerbation in children (Bosis *et al*., 2005; Freymuth *et al*., 2003; Foulongne *et al*., 2006; Jartti *et al*., 2002; Peiris *et al*., 2003; Schildgen *et al*., 2004; Williams *et al*., 2004; von Linstow *et al*., 2004) support for this association is not universal (Rawlinson *et al*., 2003). Nevertheless, wheezing is a common symptom in children with

HMPV LRTI (Chen *et al*., 2010; Døllner *et al*., 2004; Esper *et al*., 2010; García-García *et al*., 2006a; Pizzorno *et al*., 2010; Williams *et al*., 2004). The increased prevalence of asthma after hospital admission for wheezing in early childhood is well documented with HRSV the most frequently implicated (García-García *et al*., 2007). Limited data suggests that HMPV infection in infancy is one of the most significant independent factors for the development of pre-school asthma (García-García *et al*., 2007). A possible mechanism for post-bronchiolitis disease is that HMPV might persist in the lung providing a stimulus that could contribute to wheezing and asthma (Liu *et al*., 2009). It is known that acute HMPV infection in the BALB/c mouse, a representative model of HMPV infection, is associated with long-term pulmonary inflammation that leads to significant obstructive disease of the airways (Hamelin *et al*., 2006). Furthermore, primary HMPV infection elicits weak innate and aberrant adaptive immune reponses characterised by induction of a T helper (TH)2-type cytokine response at later stages of infection that coincides with increased interleukin-10 (IL-10) expression and persistent virus replication in the lung (Alvarez and Tripp, 2005). A recent study showed that HMPV mediates biphasic replication in respiratory epithelial cells and subsequently migrates to infect immune-privileged neuronal cells that innervate the lungs as a means to facilitate persistence (Liu *et al*., 2009). The implications of these findings are important in understanding HMPV disease sequelae and disease chronicity (Liu *et al*., 2009).

1.4.7.4. Other manifestations of human metapneumovirus infection

Although it is not yet confirmed there is increasing evidence that HMPV may be one causative agent rather than an innocent bystander and under hitherto unknown circumstances induces encephalitis (Schildgen *et al*., 2007). At present, definitive conclusions on possible direct effects of the virus on the central nervous system are limited to a case observation of fatal encephalitis in a child (Schildgen *et al*., 2005b). A possible etiologic relationship between HMPV and the observed neurological manifestations was based on the detection of post-mortem HMPV RNA in brain and lung tissue samples (Schildgen *et al*., 2005b). Evidence of active inflammation in both the lung and brain tissues served to complement the virological findings further (Schildgen *et al*., 2005b). Other reports of HMPV infection with potential association to central nervous infection in children have failed to implicate the virus directly as a cause of neurological manifestations (Arnold *et al*., 2009; Glaser *et al*., 2006; Kaida *et al*., 2006; Hata *et al*., 2007). However, indirect effects of infection with HMPV on the central nervous system are more widely reported (Arnold *et al*., 2008, 2009; IJpma *et al*., 2004; Kashiwa *et al*., 2004; Lau *et al*., 2006; Peiris *et al*., 2003;

Schildgen *et al*., 2005b). Indeed, Arnold *et al*., (2009) described 9 cases of neurological disease temporally associated with the presence of HMPV nucleic acid in the upper respiratory tract of children. In this study, seizures were more common in children with HMPV infection (6.3%) than those with HRSV infection (0.7%, *P*= 0.031) (Arnold *et al*., 2009). Similar findings were reported in an earlier study in which 25% of children with HMPV infection compared to 8% of children with HRSV infection suffered febrile seizures (Lau *et al*., 2006). Peiris *et al*., (2003) also reported that HMPV may be an important cause of febrile seizures following the finding that 5/32 (15.6%) HMPV infected children suffered febrile seizures compared to 1/32 (3.1%) and 3/32 (9.4%) children with HRSV and influenza infection, respectively. Some children had multiple seizures during the same episode of HMPV infection (Peiris *et al*., (2003). The increased frequency with which febrile seizures occur among patients infected with HMPV in comparison to HRSV infection serves to highlight the potential importance of HMPV in central nervous system manifestations. Moreover, HMPV infection is frequently associated with high fever (>38^{o}C) (Døllner *et al*., 2004; Kashiwa *et al*., 2004; Takao *et al*., 2003), a clinical manifestation that may be caused by direct or indirect interaction of the virus with the central nervous system (Schildgen *et al*., 2007). Indeed, HMPV infection possesses a capacity to frequently induce fever (Ali *et al*., 2010; Bosis *et al*., 2005; Choi *et al*., 2006; Døllner *et al*., 2004; Esper *et al*., 2004; Peiris *et al*., 2003; Samransamruajkit *et al*., 2006) that can exceed 39^{o}C (Peiris *et al*., 2003; Takao *et al*., 2003) and persist for longer than in children with other respiratory viruses (Peiris *et al*., 2003; Kashiwa *et al*., 2004; Wang *et al*., 2006). However, these findings failed to attain statistical significance (Arnold *et al*., 2009; Kashiwa *et al*., 2004; Peiris *et al*., 2003). Ultimately, these findings are yet unsubstantiated and remain controversial. Whether HMPV spreads beyond the respiratory system during infection, remains to be determined (Kahn, 2006).

1.4.8. Epidemiology

1.4.8.1. Geographical and Seasonal Distribution

Subsequent to the discovery of HMPV in 2001 (van den Hoogen *et al*., 2001), this novel respiratory virus has emerged as a human pathogen of worldwide importance (Figure 1.8).

Figure 1.8. Geographical distribution of human metapneumovirus.

The virus has emerged as a human pathogen exhibiting worldwide distribution following the discovery of this virus in the Middle East (Al-Sonboli *et al*., 2005; Kaplan *et al*., 2008; Malekshahi *et al*., 2010; Rashid *et al*., 2008; Regev *et al*., 2006;), Africa (Berkley *et al*., 2010; El Sayed Zaki *et al*., 2009; Fodha *et al*., 2004; Madhi *et al*., 2003), The Caribbean (Matthew *et al*., 2009) North America (Boivin *et al*., 2002; Esper *et al*., 2003), Central America (Noyola *et al*., 2005; Ulloa-Gutierrez *et al*., 2009), South America (Cuevas *et al*., 2003; Escobar *et al*., 2009; Galiano *et al*., 2004; Gray *et al*., 2006; Pizzorno *et al*., 2010), Australasia (Abdullah Brooks *et al*., 2007; Arnott *et al*., 2011b; Chen et al., 2010; Do *et al*., 2011; Ebihara *et al*., 2004b; Kim and Lee, 2005; Li *et al*., 2009; Lin *et al*., 2005; Loo *et al*., 2007; Nissen *et al*., 2002; Peiris *et al*., 2003; Rao *et al*., 2004; Thanasugarn *et al*., 2003; Werno *et al*., 2004; Xiao *et al*., 2010), United Kingdom & Europe (Antunes *et al*., 2010; Baer *et al*., 2008; Carr *et al*., 2005; Christensen et al., 2003;Freymuth *et al*., 2003; García-García *et al*., 2006; Jartti *et al*., 2002; Larcher *et al*., 2006; Ljubin-Sternak *et al*., 2008; Maggi *et al*., 2003; Pavlova *et al*., 2009; Rafiefard *et al*., 2008; Sivaprakasam *et al*., 2007; Stockton *et al*., 2002; Tecu *et al*., 2007; van den Hoogen *et al*., 2001; Viazov *et al*., 2003; von Linstow *et al*., 2004; Xepapadaki *et al*., 2004).

In temperate regions of the world, a clear seasonal variation in the incidence of respiratory tract infections exists with the peak of activity occurring in the winter months. Despite the absence of a winter season in the tropics, consistent seasons of respiratory infection, albeit less distinct, exist (Shek and Lee, 2003). The seasonality of respiratory tract viruses is exemplified by HRSV and influenza virus that primarily circulate in the winter to early spring in temperate regions and in the late spring into the summer in the subtropics. In contrast, parainfluenza viruses and rhinoviruses remain endemic within the human population throughout the year though annual peaks of infection occurring in late autumn and winter, and early autumn and spring, respectively, coincide with the respiratory season in temperate regions (Monto and Cavallaro, 1971). Initial studies to determine the temporal pattern of HMPV infection were conducted within one or more typical respiratory seasons (Bastien *et al*., 2003; Boivin *et al*., 2003; Cuevas *et al*., 2003; Esper *et al*., 2003; Falsey *et al*., 2003; Freymuth *et al*., 2003; Nicholson *et al*., 2006; Stockton *et al*., 2002; Viazov *et al*., 2003). Hence, it was impossible to determine whether HMPV exhibited seasonal peaks of activity or circulated continually throughout the year. Subsequent surveillance studies conducted over one or more consecutive years revealed a pattern of alternating years of high and low incidence with the majority of HMPV infections occurring in late winter and spring in temperate zones of the southern and northern hemisphere and often concurrently with HRSV and influenza virus (Agapov *et al*., 2006; Baer *et al*., 2008; Esper *et al*., 2004; García-García *et al*., 2006a; Kaida *et al*., 2006; Maggi *et al*., 2003; McAdam *et al*., 2004; Mullins *et al*., 2004; Oliveira *et al*., 2008; Robinson *et al*., 2005; Sloots *et al*., 2006; van den Hoogen *et al*., 2003). Although identification of HMPV infection in summer and autumn, albeit at low frequency, indicates that HMPV circulates throughout the year (Chano *et al*., 2005; Esper *et al*., 2004; Louie *et al*., 2007; McAdam *et al*., 2004; Mullins *et al*., 2004; Oliveira *et al*., 2009; van den Hoogen *et al*., 2003; Williams *et al*., 2004). In contrast, the peak of HMPV activity in a subtropical climate occurred in spring and the early summer months (Peiris *et al*., 2003; Wang *et al*., 2008). However, longitudinal studies provide the most detailed epidemiological picture to date of the seasonal distribution of HMPV infection in temperate climates (Aberle *et al*., 2008; Heininger *et al*., 2009; Rafiefard *et al*., 2008; Weigl *et al*., 2007). Indeed, these studies reveal that the epidemiology of HMPV differs markedly from other common respiratory viruses (Rafiefard *et al*., 2008) in that HMPV epidemics follow a biennial pattern of alternate winter versus spring activity (Aberle *et al*., 2008; Aberle *et al*., 2010) (Figure 1.9) that are anti-cyclical in rhythmicity to HRSV epidemics (Rafiefard *et al*., 2008; Weigl *et al*., 2007).

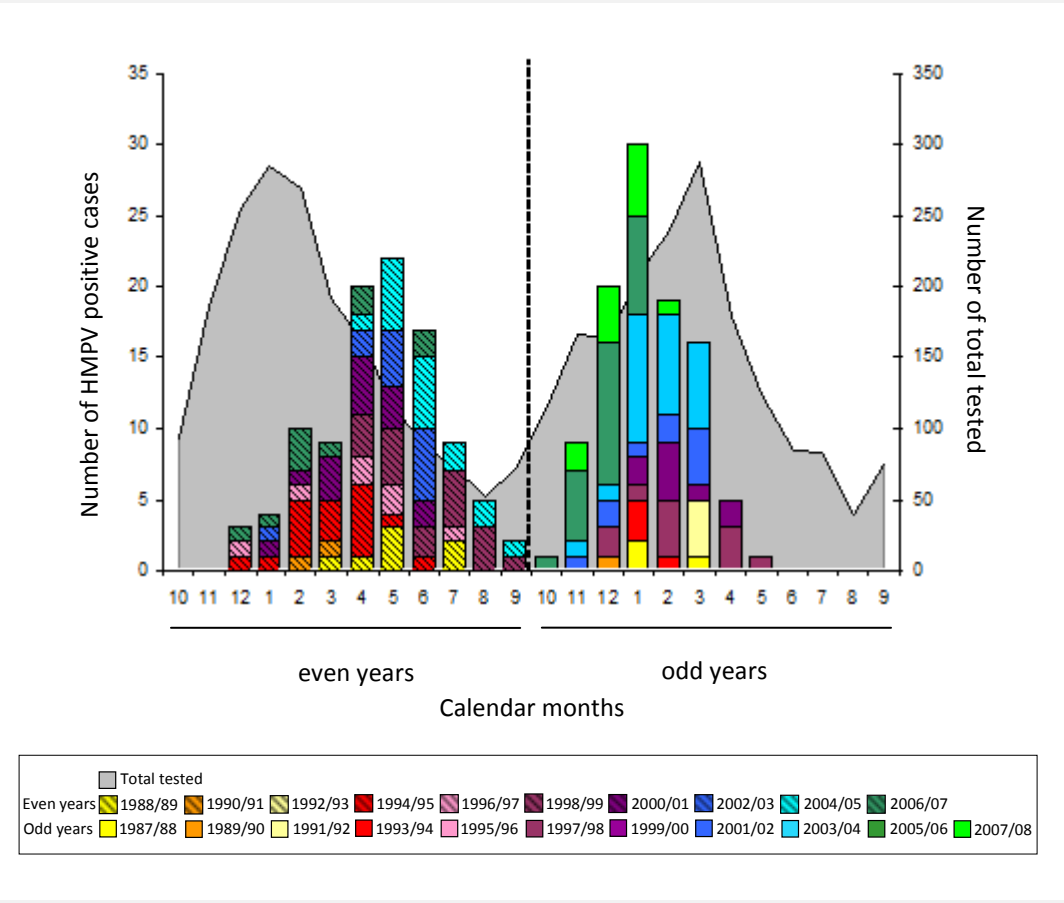


Figure 1.9. Monthly numbers of human metapneumovirus (HMPV) positive cases in even years (epidemiologic years beginning in an even year; hatched columns) and odd years (whole-coloured columns) and cumulative monthly numbers of tested samples (area under curve).

Epidemiologic year is defined as the beginning of October to the end of September of the following year. Dashed line represents change of epidemiologic years (Adapted from Aberle *et al*., 2010).

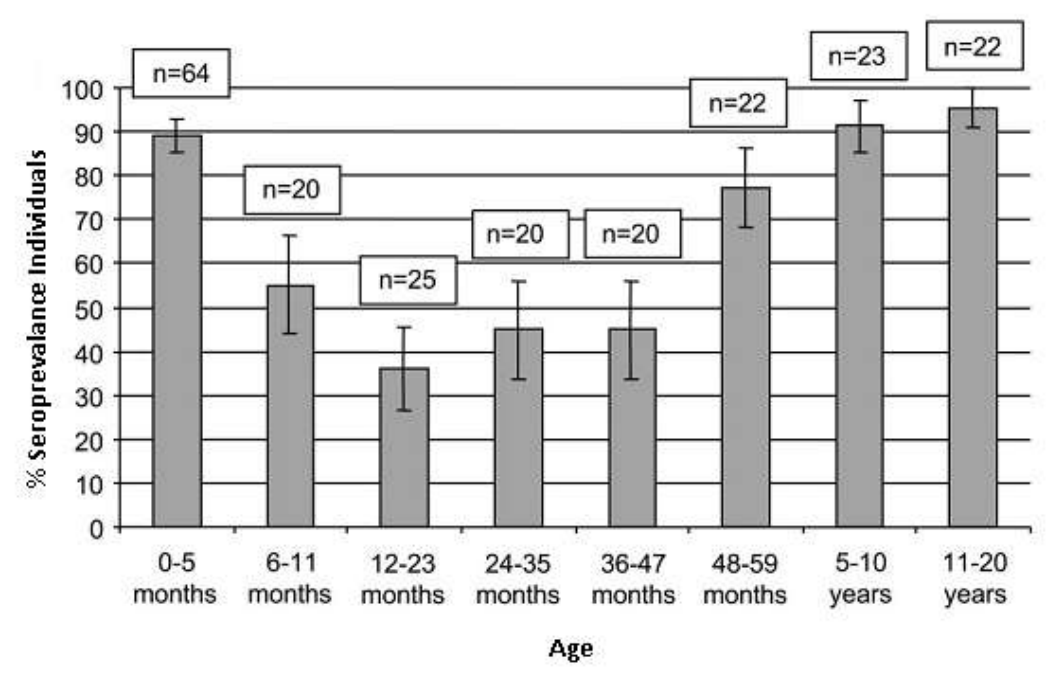
1.4.8.2. Seroepidemiology

Numerous methods have been utilised to estimate the seroprevalence of HMPV within the human population. These include indirect immunofluorescence assays using HMPV-infected cells (Boivin *et al*., 2002; Ebihara *et al*., 2003; Ljubin-Sternak *et al*., 2006; Mirazo *et al*., 2005; van den Hoogen *et al*., 2001; Wolf *et al*., 2003), enzyme-linked immunosorbent assays based on HMPV-infected cells (Falsey *et al*., 2003), specific recombinant HMPV proteins (Hamelin and Boivin, 2005; Huang *et al*., 2010; Leung *et al*., 2005; Lu *et al*., 2010; Liu *et al*., 2007b, 2010; Pavlin *et al*., 2008) or whole virus (Okamoto *et al*., 2010), and neutralisation assays (Falsey *et al*., 2009; Matsuzaki *et al*., 2008; van den Hoogen *et al*., 2001). Evidence substantiates that HMPV is a ubiquitous pathogen (van den Hoogen *et al*, 2001) since exposure to the virus is a common phenomenon (Liu *et al*., 2007b) irrespective of the methodology employed. Primary infection occurs during early childhood (Don *et al*., 2008;

Hamelin and Boivin, 2005; Leung *et al*., 2005; Ljubin-Sternak *et al*., 2006; Okamoto *et al*., 2010; van den Hoogen *et al*., 2001), albeit later than infection with HRSV (Ebihara *et al*., 2004c; Lu *et al*., 2011), and by 5 to 10 years of age virtually all children are seropositive for the virus (Don *et al*., 2008; Ebihara *et al*. 2003; Leung *et al*., 2005; Lu *et al*., 2010; Okamoto *et al*., 2010; Regev *et al*., 2006; Wolf *et al*., 2003; van den Hoogen *et al*., 2001). Exposure to HMPV during the first 2 years of life was studied by longitudinal serological analysis in 40 healthy children in Israel. By the age of 2 months, 80% children had anti-HMPV antibodies. The seropositivity rate decreased to a minimum by age 13 months and increased to 52% by age 24 months (Wolf *et al*., 2003). In another study, the percentage of children with HMPV-specific antibody was 89.1% in ≤5 months old, 55.0% in children 6 to 11 months old, 36.0% in children 12 to 23 months old, 45.0% in children 24 to 47 months old, 77.3% in children 48 to 59 months old, 91.3% in children 5 to 10 years old, and 95.5% for individuals 11 to 20 years old (Figure 1.10) (Leung *et al*., 2005).

Figure 1.10. Seroepidemiology of human metapneumovirus.

The number of serum samples tested (n) and the percentage (%) of human metapneumovirus seropositivity for each age group are indicated. Error bars represent 95% confidence intervals (Leung *et al*., 2005).



The decline in the proportion of seropositive individuals during the first year of life likely represents waning maternally acquired antibody (Leung *et al*., 2005). However, it appears that two periods of acquisition of HMPV infection in childhood also occur. The first period occurs within the first 3 years of life (Leung *et al*., 2005). During this period, the percentage

of individuals who are seropositive essentially remains constant at 35 to 45% for children 12 to 47 months of age as the decline in maternal antibody is superimposed on the increase in antibody acquired during this first period of acquisition of HMPV infection. The second period occurs in children of >48 months of age. The percentage of seropositive individuals increases to 77.3% in children aged 48 to 59 months old and to >90% in children who are >5 years old. This second peak likely reflects increased exposure to the virus, perhaps at day care or preschool environments (Leung *et al*., 2005). Ultimately, the dynamics of the seroepidemiology of HMPV within the paediatric population is complex (Leung *et al*., 2005). Despite near-universal exposure in childhood (Pavlin *et al*., 2008), re-infection can occur in all age groups throughout life (Boivin *et al*., 2007; Ebihara *et al*., 2004a; Matsuzaki *et al*., 2008; Pavlin *et al*., 2008; Williams *et al*., 2004) due to incomplete protective immunity and/or acquisition of new genotypes (Hamelin *et al*., 2004). Immunocompromised, very young, and frail elderly hosts are at highest risk of serious sequelae as a result of HMPV re-infection (Boivin *et al*., 2002; Boivin *et al*., 2007; Falsey *et al*., 2003; Martinello *et al*., 2006; Martino *et al*., 2005; Pelletier *et al*., 2002). In a recent study, it was suggested that antibody may play a role in protection from infection with HMPV since serum antibody levels were significantly lower in adults who subsequently became infected with HMPV compared to those who remained infection free (Falsey *et al*., 2010).

1.4.8.3. Clinical epidemiology

The relationship between disease severity and genetic variability of HMPV is a clear source of debate (Arnott *et al*., 2011b; Ljubin-Sternak *et al*., 2008; Pitoiset *et al*., 2010; Vicente *et al*., 2006; Williams *et al*., 2004). Indeed, the possibility that HMPV genotypes differ in virulence is intriguing (Esper *et al*., 2004). Epidemiologic studies report the general predominance of HMPV genotype A within the human population and certainly this is reflected in the increased prevalence of this genetic lineage in hospitalised children (Caracciolo *et al*., 2008; Esper *et al*., 2004; Loo *et al*., 2007; Manoha *et al*., 2007; Noyola *et al*., 2005; Vicente *et al*., 2006). However, the consequence of the higher circulation frequency of HMPV genotype A is perhaps more sinister since it is suggested that infection with lineage A is associated with greater disease severity (Kaida *et al*., 2006; Vicente *et al*., 2006). Indeed, diagnosis of pneumonia was more common and the severity index that combined hospital admission, intensive care unit stay, and oxygen saturation <90% was higher for patients with HMPV genotype A infection (Vicente *et al*., 2006). In contrast, frequency of laryngitis was higher in children with a genotype B1 HMPV infection whereas

wheezing occurred more often in association with genotype B1 and B2 HMPV infections suggesting that differences in pathogenesis may be dependent on HMPV genotype (Matsuzaki *et al*., 2008). Nevertheless, other studies reported no clinical differences in the severity of disease according to genotype (Agapov *et al*., 2006; Bosis *et al*., 2008; Gaunt *et al*., 2009; Legrand *et al*., 2011; Manoha *et al*., 2007; Pitoiset *et al*., 2010; Williams *et al*., 2004; Xiao *et al*., 2010). Bosis *et al*., (2008) found no differences in disease presentation or in clinical or socioeconomic impact in relation to viral genotypes. However, HMPV viral load was significantly higher in children with lower respiratory tract involvement ($p<0.05$), hospitalised children ($p<0.05$), and the prevalence of secondary cases of a similar disease in the household of index cases ($p<0.05$) (Bosis *et al*., 2008). It is clear that the relationship between disease severity and HMPV genotype remains unresolved at present and the dynamics of HMPV infection are clearly multifactoral.

CHAPTER TWO

2. General material and methods

2.1. Aims

This chapter outlines the analytical methodology and the materials that were employed in the routine diagnosis of respiratory tract infection and the development and implementation of strategies for the detection of HMPV within the Microbiology Department at the Norfolk and Norwich University Hospital, which was just beginning to develop a molecular diagnostic service. The prepondance of analytical methodology and the materials described were followed in subsequent experiments.

2.2. Safety

All laboratory procedures that necessitated the manipulation of clinical samples with the resultant generation of infectious aerosols were conducted within a certified class I Biological Safety Cabinet (BSC). A laboratory coat and disposable gloves were worn during all procedures. All work surfaces were decontaminated after any spill of potentially dangerous material and at the end of the working day.

2.3. Study Population

The Norfolk and Norwich University Hospitals NHS Foundation Trust provides Tertiary Services to a total population of 822,500 and Secondary Services to a catchment of 654,900 across Norfolk and North Suffolk. It is an Acute Teaching Trust providing comprehensive General and Specialist Services on two sites: the 1,000-bed Norfolk and Norwich University Hospital and Cromer and District Hospital on the North Norfolk Coast.

From 31st October 2005 and 31st December 2008, 1,536 NPA samples collected from children $\leq$18 years of age attending the Norfolk and Norwich University Hospital with acute respiratory symptoms were submitted to Microbiology Department for routine investigations for microbial causes of ARTI. The samples series amassed for archival storage consisted of 821 NPA samples; insufficient residual volume was available for a proportion of the remaining samples while other samples were not retained beyond the normal storage period associated with routine diagnostic practice.

A second set of respiratory specimens were collected as part of the national Weekly Returns Service (WRS) influenza sentinel practice network of the Royal College of General

Practitioners (RCGP) from patients that presented with influenza-like illness (ILI) or other acute respiratory illness.

Other respiratory specimen types that included sputum, bronchoalveolar lavage (BAL), and lung or endotracheal aspirate specimens as well as combined nose and throat swabs and throat swabs that were not collected as part of the national virological influenza surveillance scheme were selected for conducting evaluations of new diagnostic equipment or tests.

2.4. Ethical Approval

Permission to undertake retrospective investigations for HMPV on residual NPA samples was granted by the East Norfolk and Waveney Research Governance Committee and the Norfolk Research Ethics Committee (REC Reference number: 05/Q0101/77) (Appendix I).

2.5. Routine Investigations for Causes of Respiratory Tract Infection

2.5.1. Bacteriology

The isolation of bacteria known to cause respiratory tract infection was performed in accordance with the National Standard Method (NSM) Bacteriology Standard Operating Procedure (BSOP) 57 entitled "Investigation of Bronchoalveolar Lavage, Sputum, and Associated Specimens" and Quality Standard Operating Procedure (QSOP) 52 entitled "*Inoculation of Culture Media*" (HPA, 2008a) issued by the Standards Unit, Department for Evaluations, Standards and Training (DEST), Centre for Infections (Cfl), Health Protection Agency (HPA).

Mucoid material was first treated with Sputasol (Oxoid Ltd, Hampshire, UK), a freeze-dried supplement containing the mucolytic agent, dithiothreitol, by the addition of an equal volume of a prepared 0.1% solution of the supplement to the sample material. The mixture was subjected to vigorous agitation and finally incubated at 37°C, with periodic shaking, until liquefaction was complete. Non-mucoid samples and treated mucoid samples were inoculated onto Columbia agar base supplemented with 5% defibrinated horse blood, Columbia Agar base supplemented with chocolated horse blood and cystine lactose electrolyte deficient (CLED) agar (Oxoid Ltd, Hampshire, UK) using a sterile 10 µl loop. The inoculum was dispersed over the surface of each agar plate by the surface streak method in order to achieve the isolation of individual bacterial colonies. Blood agar plates were supplemented by the addition of an optochin disc (Oxoid Ltd, Hampshire, UK) to aid

differentiation between *Streptococcus pneumoniae* and other α-haemolytic *Streptococcus* species. A 10 µg bacitracin disc (Oxoid Ltd, Hampshire, UK) was placed on the chocolate agar to aid screening for *Haemophilus influenzae*. Both antimicrobial discs were placed near the edge of the plate between the area covered by the first and second spread to avoid total inhibition of very susceptible organisms (HPA, 2008a). The inoculated agar plates were incubated at 35-37°C in air enriched with 5-10% CO_2 for 48 hours. The plates were read daily. The quantity of bacteria in culture was semi-quantitatively defined as low (+), moderate (++) or high (+++). Bacteria considered potential pathogens in NPA samples were *S. pneumoniae*, *H. influenzae*, *Moraxella catarrhalis,* and *Staphylococcus aureus*; other organisms grown in pure growth could be considered significant. A mixed growth of upper respiratory tract flora was defined as no significant growth (NSG).

2.5.2. Virology

Liquid specimens including NPA, endotracheal aspirates, sputum, and BAL were tested for respiratory pathogens by a combination of viral culture and DFA (IMAGEN™, Oxoid Ltd, Hampshire, UK) while the BinaxNOW® RSV test (Binax, Inc., ME, USA) was applied to the investigation of NPA samples only. Viral culture alone was performed on swab specimens due to the absence of adequate cellular material for DFA.

2.4.2.1. BinaxNOW® RSV Test

This rapid immunochromatographic membrane assay for the qualitative detection of HRSV F protein antigen in NPA samples was performed as a rapid point of care screening test within the acute hospital setting as a useful adjunct to the diagnosis of HRSV infections in symptomatic neonatal and paediatric patients under the age of five years. Test positive and negative control swabs were provided once with each kit. The control swabs were used on first use of a new kit to ensure that the test reagents were working.

2.4.2.2. Preparation of Clinical Material

Successful recovery of a virus from clinical material depends on the quality of material received for inoculation (HPA, 2010a). Many viruses are susceptible to drying, adverse pH, and varying osmotic potential (HPA, 2010a). Hence, samples were placed in virus transport medium (VTM) as soon as possible after collection.

Nasopharyngeal aspirate and other liquid specimen types

On receipt, all liquid specimens were mechanically homogenised by vortexing for 15 seconds to decrease the viscosity of the samples. Approximately 500 µl of each sample was transferred into a labelled VTM for routine virus isolation. An additional 1 to 2 ml of material taken directly from the aspirate was transferred to a labelled universal for the preparation of slides for DFA test. Briefly, 10 ml of phosphate buffered saline (PBS) (Appendix II) was added to the universal. The resultant cell suspension was centrifuged at room temperature (15^{o}C to 25^{o}C) at 380x*g* for 10 minutes and the supernatant was removed and discarded. The cell pellet was re-suspended in 200 µl of PBS in order to dilute any remaining mucus whilst maintaining a high cell density for the preparation of slides for direct immunofluorescence test. The presence of excess mucus prevents adequate penetration of DFA staining reagents with resultant non-specific fluorescence and so to aid clarification of the cells the wash step was repeated until all mucoid material was removed.

Swabs

Swab specimens received in VTM were vortexed for 15 seconds to dislodge material on the swab into the transport medium.

2.4.2.3. Direct immunofluorescence

This rapid diagnostic test was used for the direct detection of influenza virus types A and B, PIV types 1-3, HRSV, adenovirus, and *Chlamydia* sp. in clinical specimens and confirmation of the presence of these respiratory pathogens in cell culture. It was performed using the IMAGEN^{TM} immunofluorescence test (DFA, IMAGEN^{TM}, Oxoid Ltd, Hampshire, UK), a commercial qualitative immunofluorescence test that contains monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC). All methodology practised within the Microbiology Department reflect NSM Virology Standard Operating Procedure (VSOP) 22 entitled "*Immunofluorescence and Isolation of Viruses from Respiratory Samples*" (HPA, 2007) issued by the Standards Unit, DEST, Cfl, HPA. Positive control slides were used to check that the staining procedure had been satisfactorily performed. If a negative control was required, uninfected intact cells of the type used for the culture and isolation of respiratory viruses were used.

2.4.2.4. Isolation of Viruses from Respiratory Samples

The purpose of virus isolation is to demonstrate the presence and viability of viruses in clinical specimens (HPA, 2007). All methodology practised within the Microbiology Department reflect NSM VSOP 22 entitled "*Immunofluorescence and Isolation of Viruses from Respiratory Samples*" (HPA, 2007) and NSM VSOP 39 entitled the "*Procedure for the care and propagation of cell cultures for virus isolation*" (HPA, 2009) issued by the Standards Unit, DEST, Cfl, HPA. All NPA samples were inoculated onto the following continuous cell culture monolayers: HEp-2, human hepatoma (PLC-PRF5), and normal human foetal lung fibroblast (MRC-5). Uninoculated HEp-2, PLC-PRF5 and MRC-5 cell culture monolayers containing maintenance medium only served as controls and were incubated alongside inoculated cell culture monolayers. The appearance of inoculated cell culture monolayers was compared to negative control monolayers when the tubes were observed under light microscopy. HEp-2, PLC-PRF5, and MRC-5 cell culture monolayers were not inoculated with respiratory viruses to serve as positive controls.

Cell culture monolayers were supplied ready to use, in disposable glass tubes, by the Clinical Microbiology and Public Health Laboratory (CMPHL), Addenbrooke's Hospital, Cambridge. The tubes were incubated at 33^{o}C for 16 days on a roller drum. The tubes were observed every 24 or 48 hours to check for the development of CPE and contamination under light microscopy. Sub-passage was only necessary if cell cultures were contaminated with bacteria or fungi or displayed degeneration of the cell sheet. Detection of viruses that did not produce CPE was performed by haemadsorption. All methodology practised within the Microbiology Department reflect NSM VSOP 45 entitled "*Haemadsorption of Viruses*" (HPA, 2010a) issued by the Standards Unit, DEST, Cfl, HPA.

2.6. Specimen Storage

On completion of routine investigations for microbial causes of respiratory tract infection all residual nasopharyngeal aspirate samples were divided into aliquots, labelled and stored at 2°C to 8°C for no longer than 24 hours or frozen at −70°C for prolonged storage.

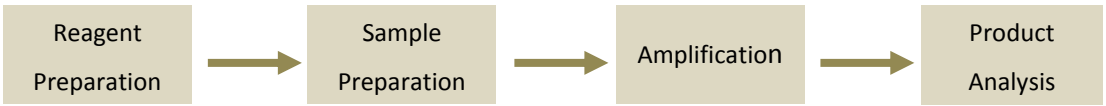
2.7. Molecular Diagnostics

2.7.1. Good Laboratory Practice when Performing Molecular Amplification Assays

At the start of this research degree programme the molecular diagnostic service within the Microbiology Department of the Norfolk and Norwich University Hospital was in its infancy. Careful consideration was given to facility design and operation within the Virology Laboratory in which nucleic acid amplification methods were to be performed. All issues were considered with reference to the NSM QSOP 48 entitled "*Good Laboratory Practice When Performing Molecular Amplification Assays*" (HPA, 2010b) issued by the Standards Unit, DEST, Cfl, HPA.

2.6.1.1. Organisation of Work

A major problem associated with nucleic acid amplification methods is the potential for cross-contamination leading to the generation of false positive results. A widely adopted solution to this universal problem has been to perform each individual stage within a separate room. Thus, three discrete areas/rooms were dedicated to the reagent preparation, sample preparation, PCR amplification, and analysis of PCR products. Workflow between these areas/rooms was unidirectional:



A dedicated set of pipettes was provided in each area for use with filter tips to reduce aerosols. Disposable gloves were worn at all times to avoid introducing contamination from RNases found on most human hands. These were changed whenever suspected of contamination and before moving to the next work area. Dedicated laboratory coats, workbooks, and stationary were supplied for use in each area/room. All reagents and consumables used were RNase-free to avoid degradation of viral RNA by RNases.

2.6.1.2. Reagent Preparation – The Clean Room

It was essential that this area remained free of extraneous nucleic acid to avoid the cross-contamination of the reagents stored here. Reagent preparation was performed to the highest standards of aseptic technique. Where possible, batches of reagents were aliquoted to protect stock reagents from contamination and to minimise the potential consequences should contamination occur. Furthermore, by aliquoting reagents any detrimental effects

resulting from excessive freeze thawing were avoided. All aliquots were clearly labelled and stored separately from all other stock.

2.6.1.3. Sample Preparation

Nucleic acid extraction is the first step in the detection of a potential target pathogen in clinical specimens and therefore of great importance since it can influence the success of downstream applications. The provision of a separate dedicated area was thus required for this purpose.

2.6.1.4. The Amplification Room

Only essential items were taken into the amplification room. All outer packaging was removed before items were taken through to this room. Firstly, this was to prevent contamination being introduced into the room and secondly to avoid the task of disposing of the packaging safely. To avoid specimen contamination from seepage into poorly capped tubes, water baths were avoided; dry baths or dry heat blocks, if needed, were preferable.

2.6.1.5. The Product Analysis Room

This was designated as a contaminated area. To prevent the escape of amplicons into the environment, strict anti-contamination measures were applied in this room. No reagents or equipment was removed from this room at any time.

2.6.1.6. Glassware

Glassware was baked in an oven at 450°F for six to eight hours to eliminate potential RNase contamination. Prior to baking, beakers and flask tops were wrapped with aluminium foil to prevent contamination after baking. Treated items were marked as "RNase-free" to distinguish them from untreated items and stored in a clearly marked "RNase-free Zone" to prevent accidental contamination.

2.6.1.7. Disposable plasticware

The use of certified non-pyrogenic, DNase- and RNase-free consumables including tubes and filter pipette tips was maintained throughout procedures.

2.6.1.8. Non- disposable plasticware

To ensure all non-disposable plasticware was free of extraneous nucleic acid prior to use, it was treated with 1,000 ppm chlorine solution that was prepared by combining 1 x 5.0 g actichlor effervescent chlorine releasing tablet (VWR International Ltd, Dublin, Ireland) in 1 litre of water. Non-disposable plasticware was rinsed thoroughly with distilled water to remove residual chlorine solution.

2.6.1.9. Preparing RNase-free Solutions

DEPC-treatment is the most common method used to inactivate RNases in water and buffers. However, DEPC will react with reagents containing primary amine groups including Tris and so cannot be used to treat TE buffer. As an alternative to DEPC-treatment, RNA*secure* (Applied Biosystems, Warrington, UK) a broad-spectrum RNase inhibitor, was used. A unique feature of RNA*secure* is that it can be re-heated after the initial treatment to reactivate the RNase-destroying agent and eliminate any newly introduced contaminates.

2.6.1.10. RNA Samples

As it was possible that small amounts of RNases were co-purified with isolated RNA an RNase inhibitor, SUPERase-In (Applied Biosystems, Warrington, UK) was added to all RNA samples prior to down-stream applications. SUPERase-In RNase inhibitor is a protein based inhibitor of non-human origin that noncovalently binds and inhibits the most common and troublesome RNases including RNase A, B, C, 1 and T1.

2.6.1.11. Master Mix Preparation

Master mixes were prepared on ice to avoid premature cDNA synthesis at incorrect temperatures. Mastermixes containing fluorophores were not exposed to excessive light to minimise degradation by photobleaching.

2.6.1.12. Other Approaches to Contamination Prevention

A number of additional approaches were implemented to minimise the likelihood of contamination. To eliminate deoxyribonucleic acid (DNA) contamination, decontamination of laboratory bench surfaces and equipment was performed daily with 1,000 ppm chlorine solution or a commercial, ready to use surface decontaminant called “DNA Away” (VWR International Ltd, Dublin, Ireland) that was suitable for equipment that was prone to

corrosion. All tubes were pulse centrifuged before opening to prevent aerosols. Commercial mastermixes containing Uracil-N-glycosylase (UNG) offered further precaution against potential contamination through elimination of carry-over PCR products from previous amplification experiments but not template DNA. However, master mixes incorporating UNG were not utilised in any PCR systems employed due to the association of UNG with decreased PCR sensitivity (Pang *et al*., 1992).

2.7.2. Design of primer and probe sequences

Primer and probe sequences were designed from nucleotide sequences in FASTA format, which were obtained from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments were generated using the BioEdit software package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Primer and probe sequences were generated from the nucleotide alignment profile using Primer Express Software version 2.0 (Applied Biosystems™, Warrington, UK) using parameters that met primer and probe design guidelines outlined in Table 2.1. The specificity of the primers was checked using the BLAST algorithm available from the NCBI, which provides sequence similarity searches of databases maintained by the NCBI, European Molecular Biology Laboratory (EMBL), and the DNA DataBank of Japan (DDBJ).

2.7.3. Preparation of customised primer and probe sequences

In general, customised oligonucleotide sequences were purchased commercially from Eurofins MWG Operon, Germany (www.eurofinsdna.com/) or Metabion GmbH, Germany (www.metabion.com/). An exception was the TaqMan® MGB™ or minor groove binder probes. These dual-labelled probes were purchased from Applied Biosystems™, Warrington, UK. The customised primer and probe sequences were ordered using convenient online ordering systems. All TaqMan® or TaqMan® MGB™ probes were labelled with a fluorescent reporter dye at the 3' end and a compatible non-fluorescent quencher molecule at the 5' end. Compatible combinations of reporter and quencher dyes were selected when more than one target was amplified and detected in the same PCR reaction.

Table 2.1. Primer and probe sequences generated from the nucleotide alignment profile using Primer Express Software version 2.0 using recommended guidelines for the probe (top) and primers (bottom).

Amplicon Length	50 to 150 bases for optimum PCR efficiency
Probe Length	13 to 30 bases if using conventional TaqMan probes
Melting Temperature (T_m)	68 –70°C
%GC	30 – 80%
5' end	Avoid 'guanine' (G) residues. A 'G' residue adjacent to the reporter dye will quench the reporter fluorescence.
Repeating nucleotides	Avoid runs of an identical nucleotide. This is especially true for 'G' residues. If repeats are present, there must be fewer than four consecutive 'G' residues.
Consecutive A residues	Avoid six consecutive 'adenine' (A) residues anywhere in the probe.

Melting Temperature (T_m)	58 –60°C
%GC	30 – 80%
3' end	The last 5 nucleotides at the 3' end should contain no more than 2 'G' and/or 'cytosine' (C) residues
Repeating nucleotides	Avoid runs of an identical nucleotide. This is especially true for 'G' residues. If repeats are present, there must be fewer than four consecutive 'G' residues.

All oligonucleotides were selected for preparation at a synthesis scale of 0.2 µmol with exclusion of TaqMan® MGB™ probes, which were selected at synthesis scale of 20,000 pmol. The primers were synthesised by High Purity Salt Free purification technology (HPSF). This technology is based on liquid chromatography. It has been developed especially for unmodified and oligonucleotides with 15 to 50 bases in length. High Performance Liquid Chromatography (HPLC) or Reverse Phase High Performance Liquid Chromatography (RP-HPLC) was used for purifying all oligonucleotide probes. All oligonucleotides were supplied lyophilised with the exclusion of TaqMan® MGB™ probes and were reconstituted with Tris-EDTA (TE) Buffer (Ambion®, Warrington, UK) consisting of 10 mM Tris (tris(hydroxymethyl)aminomethane), adjusted to pH 8.0 with hydrochloric acid (HCl), and 1 mM EDTA (ethylenediaminetetraacetic acid). A synthesis report was provided with the appropriate volume of TE Buffer to add to each oligonucleotide to obtain a standard stock concentration of 100 µM. TaqMan® MGB™ probes were received in liquid format in 1XTE Buffer at a concentration of 100 µM. All reconstituted primers and probes were diluted further in TE Buffer, pH 8.0 to obtain a working stock concentration of 20 µM and 10 µM, respectively. This was calculated using the formula: $C_1V_1 = C_2V_2$ (Figure 2.1).

C_1 = Initial Concentration of solution

V_1 = Initial Volume of solution

C_2 = Final Concentration of solution

V_2 = Final Volume of solution

For Example: a Molar concentration is one mole of solute in one litre of solution. Therefore, a 100 µM solution would be 100 µmol in one litre. There is 1,000,000 µl in 1 L.

$$\left(\frac{100\ \mu\text{mols}}{1{,}000{,}000\ \mu\text{l}}\right)\left(50\ \mu\text{l}\right) = \left(\frac{10\ \mu\text{mols}}{1{,}000{,}000\ \mu\text{l}}\right)\left(V_2\right) \quad \text{So, } V_2 = 500\ \mu\text{l}$$

To achieve a final probe volume of 500 µl, add 450 µl of 1X TE to 50 µl of the 100 µM probe standard stock solution.

Figure 2.1. Calculation of the working stock concentration of reconstituted oligonucleotides.

All oligonucleotides were stored at -20°C until required. Probes were protected from light to minimise bleaching of the fluorescent dye. Oligonucleotides in current use were stored at 2-8°C to avoid repeated freeze–thawing cycles. Oligonucleotides were thawed no more than three times to ensure oligonucleotide integrity.

2.7.4. Purification of viral RNA

The extraction and purification of nucleic acid from clinical specimens is the obligatory but critical first step in the detection of any pathogen that may be present (Clewley, 1999). The method chosen should consist of as few steps as possible in order to reduce the chance of contamination with exogenous DNA or RNA, or, in the case of RNA extraction, exogenous RNases. There is also the potential for the loss of target nucleic acid in all protocols and this is increased in procedures that are complex with multiple steps (HPA, 2008b).

2.6.4.1. Preparation of nasopharyngeal aspirate samples and other sample types

Residual clinical specimens were fresh or frozen. However, if frozen, the samples were thawed no more than once since cryoprecipitates accumulate when samples are subjected to repeated freeze–thawing cycles leading to reduced viral titres. Samples were mechanically homogenised by vortexing for 15 seconds to decrease the viscosity of clinical material prior to sample processing.

2.6.4.2. Selection of controls for nucleic acid isolation

Controls were included in all procedures designed for the preparation of nucleic acid to verify the success of the extraction procedure and safeguard against contamination. All extraction controls were processed alongside unknown clinical samples. In the absence of a commercial working reagent for HMPV and preceding identification of positive samples by the selected real-time RT-PCR assay, positive virus culture supernatant kindly donated by Dr Terry Collins, Specialist Virology Centre, Gartnavel General Hospital, Glasgow, Scotland served as the extraction positive control in the interim. A negative VTM served as the negative extraction control.

2.6.4.3. Manual purification of viral RNA

Manual purification of viral RNA was achieved using the QIAamp Viral RNA Mini Kit (QIAGEN Ltd, West Sussex, UK), an established general-purpose commercial technology for the isolation of RNA from a wide variety of viruses, in accordance with the manufacturer's instructions. Briefly, 560 µl of prepared Buffer AVL containing carrier RNA was added to a 1.5 ml microcentrifuge tube (Alpha Laboratories, Hampshire, UK). Next, 140 µl of sample material and when applicable, 20 µl of internal control was added to the Buffer AVL-carrier RNA in the microcentrifuge tube and mixed by pulse-vortexing for 15 seconds to yield a homogenous solution. The mixture was incubated for 10 minutes at room temperature (15^{o}C to 25^{o}C). Lysis of sample material under the highly denaturing conditions provided by guanidine salts and detergent in Buffer AVL ensured inactivation of RNases and isolation of intact viral RNA. Next, 560 µl of ethanol (96–100%) was added to the 1.5 ml microcentrifuge tube and mixed by pulse-vortexing for 15 seconds. After mixing, the 1.5 ml microcentrifuge tube was centrifuged briefly to remove droplets from the inside of the lid. A spin column was placed in a 2 ml collection tube. Carefully, 630 µl of the mixture was applied to the spin column without wetting the rim. The cap was closed and the spin column was centrifuged at 8000 revolutions per minute (rpm) for 1 minute. Finally, the spin column was placed into a clean 2 ml collection tube, and the collection tube containing the filtrate discarded. The remaining mixture was added to the spin column without wetting the rim. Again, the cap was closed and the spin column was centrifuged at 8000 rpm for 1 minute. The spin column was placed into a clean 2 ml collection tube, and the collection tube containing the filtrate discarded. Buffer AVL in combination with ethanol created optimum binding conditions for the viral RNA before loading the sample onto the spin column while carrier RNA, added to Buffer AVL, improved the binding of viral RNA to the silica membrane, and reduced the

chance of viral RNA degradation. Viral RNA was absorbed onto the silica membrane during the two brief centrifugation steps while salt and pH conditions in the lysate ensured that protein and other contaminants, which can inhibit downstream enzymatic reactions, were not retained on the membrane. Viral RNA was bound to the silica-gel membrane while contaminants were washed away efficiently during the next two short centrifugation steps using two different ethanol-based wash buffers, AW1 and AW2. The spin column was opened carefully and 500 µl of Buffer AW1 was added without wetting the rim. The cap was closed and the spin column was centrifuged at 8,000 rpm for 1 minute. The spin column was placed into a clean 2 ml collection tube and the collection tube containing the filtrate discarded. The spin column was carefully opened and 500 µl of Buffer AW2 was added without wetting the rim. The cap was closed and the spin column was centrifuged at 14,000 rpm for 3 minutes. To eliminate any chance of possible Buffer AW2 carryover, the spin column was placed into a clean 2 ml collection tube, and the collection tube containing the filtrate discarded. A final centrifugation step at 14,000 rpm for 1 minute was performed to eliminate all traces of residual Buffer AW2 in the eluate to avoid potential problems in downstream applications. The spin column was placed in a clean 1.5 ml microcentrifuge tube and the collection tube containing the filtrate was discarded. Finally, RNA was eluted in Buffer AVE, a RNase-free water that contains 0.04% sodium azide to prevent microbial growth and subsequent contamination with RNases. The spin column was opened carefully and 40 µl of Buffer AVE, equilibrated to room temperature (15^{o}C to 25^{o}C), was added. The cap was closed and the spin column was incubated at room temperature (15^{o}C to 25^{o}C) for 1 minute. The spin column was centrifuged at 8000 rpm for 1 minute. To increase the yield of viral RNA, a second elution with Buffer AVE was performed. The spin column was carefully opened and 40 µl of Buffer AVE was added. The cap of the spin column was closed and then incubated at room temperature (15^{o}C to 25^{o}C) for 1 minute. The spin column was centrifuged at 8000 rpm for 1 minute. At the end of the viral RNA purification procedure, the spin column was discarded. The eluted RNA was retained in the 1.5 ml microcentrifuge tube. Viral RNA was then ready for direct use or prolonged storage at –20^{o}C.

2.6.4.4. Automated Purification of Viral RNA

The COBAS® AmpliPrep Total Nucleic Acid Isolation (TNAI) Kit (Roche Diagnostics Ltd, Burgess Hill, UK) was utilised for the preparation of highly purified total nucleic acid on the COBAS® AmpliPrep Instrument (Roche Diagnostics Ltd, Burgess Hill, UK) (Figure 2.2), in accordance with the manufacturer's instructions, to allow complete automation of the

sample preparation process based on Magnetic Glass Particle (MGP) technology. Briefly, 350 µl of clinical material was transferred manually into barcode labelled sample input tubes (S-tubes). Sample racks filled with S-tubes were loaded onto the COBAS® AmpliPrep Instrument together with ready to use barcode labelled reagent cassettes and generic consumables.

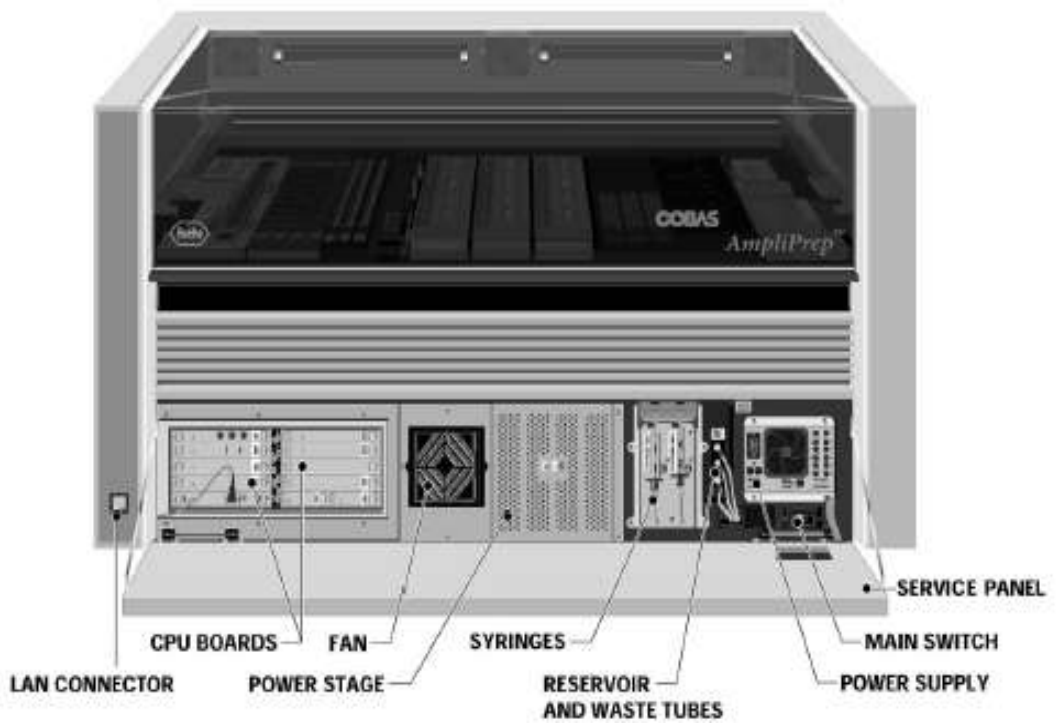


Figure 2.2. The COBAS® AmpliPrep Instrument in combination with the COBAS® AmpliPrep Total Nucleic Acid Isolation Kit for fully automated sample preparation using a generic silica-based capture technique.

When applicable, an appropriate dilution of internal control was prepared and loaded onto the COBAS® AmpliPrep Instrument with the reagent cassettes. On board the COBAS® AmpliPrep Instrument, sample processing was performed in a disposable sample-processing unit (SPU). The process commenced with the automatic addition of specimen diluent to each sample up to a final volume of 850 µl followed by protease solution, which digested proteins to facilitate the release of RNA and DNA while the addition of lysis reagent, containing guanidine salts, to the samples resulted in a complete lysis by denaturation of proteins. RNA and DNA were released and simultaneously stabilised. The released nucleic acids were bound to the silica surface of added magnetic glass particles due to chaotropic salt conditions and the high ionic strength of the lysis reagent. Next, wash reagent removed unbound substances and impurities such as denatured proteins, cellular debris, and potential PCR inhibitors such as haemoglobin and reduced the salt concentration. Finally, purified nucleic acids were eluted at elevated temperatures. Eluated nucleic acids were

available for immediate use in further downstream applications or stored at –20°C until required.

2.7.5. Real-time reverse-transcription polymerase chain reaction

Real-time PCR permits the detection of a specific PCR product as it accumulates during PCR rather than by measuring the amount of accumulated PCR product at the end of the PCR process (Applied Biosystems, 2005) by means of a fluorogenic nucleic acid probe that targets the product between the amplimer binding regions (Lee *et al*., 2009). A variety of fluorogenic probe chemistries is available for real-time PCR. These include molecular beacon, eclipse, hybridisation, and hydrolysis probes. Constructed with a fluorescent reporter dye covalently bound to the 5´ end and a quencher dye on the 3´ end, a hydrolysis probe utilises the 5´to 3' exonuclease activity of *Taq* DNA polymerase for fluorescence detection (Applied Biosystems, 2005) (Figure 2.3).

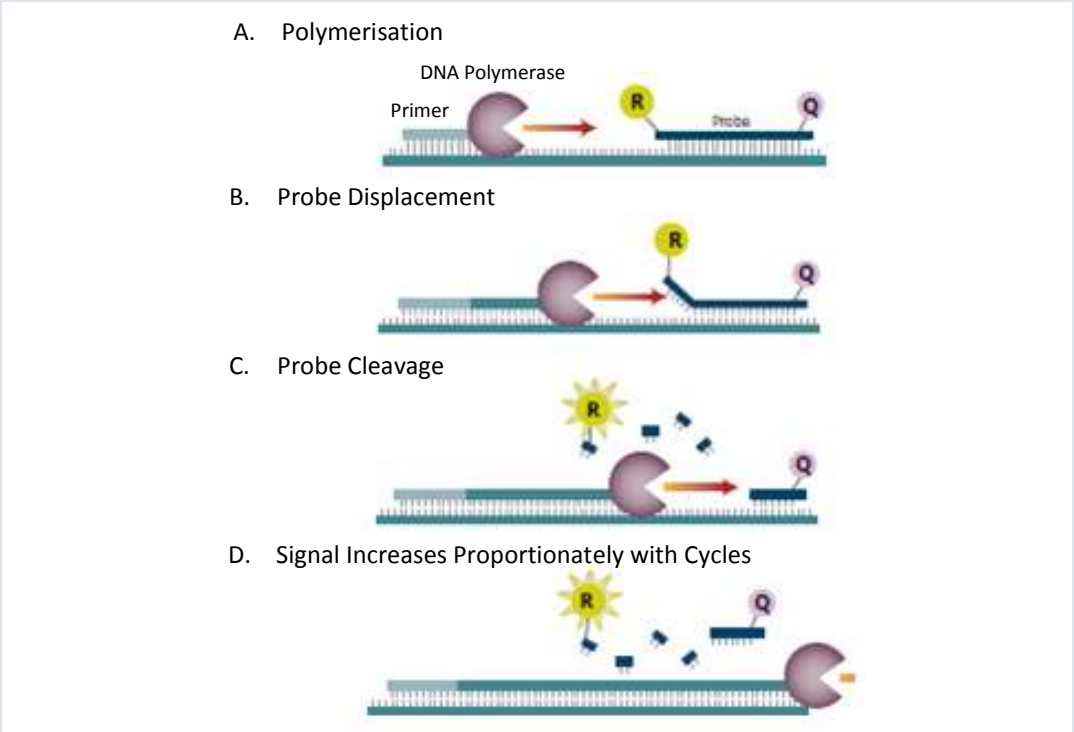


Figure 2.3. TaqMan hydrolysis probe.

(A) The probe anneals to the target sequence downstream from the primers. (B) The exonuclease activity of thermostable Taq polymerase cleaves the hybridised probe as the primer extends. (C) This cleavage serves to separate the detectable reporter fluorophore (R) from the quencher (Q). (D) This increases the fluorescence signal proportional to the number of amplicons generated. Adapted from Smart Note 6.1: Designing Real-Time Assays on the SmartCycler® II System. Available from URL: http://www.cepheid.com/media/files/smart-notes/SmartNote6.1.pdf. Accessed 12th January 2011.

While the probe is intact, the proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET; Förster Resonance) through space. If the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5´ nuclease activity of *Taq* DNA polymerase as this primer is extended. This cleavage of the probe separates the reporter dye from the quencher dye, increasing the reporter dye signal and removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. Thus, inclusion of the probe does not inhibit the overall PCR process. Additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced (Applied Biosystems, 2005). All real-time RT-PCR was performed using either the Rotor-Gene™ 6000 series real-time analyser (Corbett Research Ltd, Cambridge, UK) or the Applied Biosystems™ (ABI) Prism® 7700 Real-Time Sequence Detection System (Applied Biosystems™, Warrington, UK).

2.6.5.1. Rotor-Gene™ 6000 series real-time analyser

The Rotor-Gene™ 6000 series real-time analyser (Corbett Research Ltd, Cambridge, UK) (Figure 2.4) uses a unique centrifugal rotary format to ensure optimal thermal and optical uniformity between samples. Samples spin continually at 400 rpm during each run.

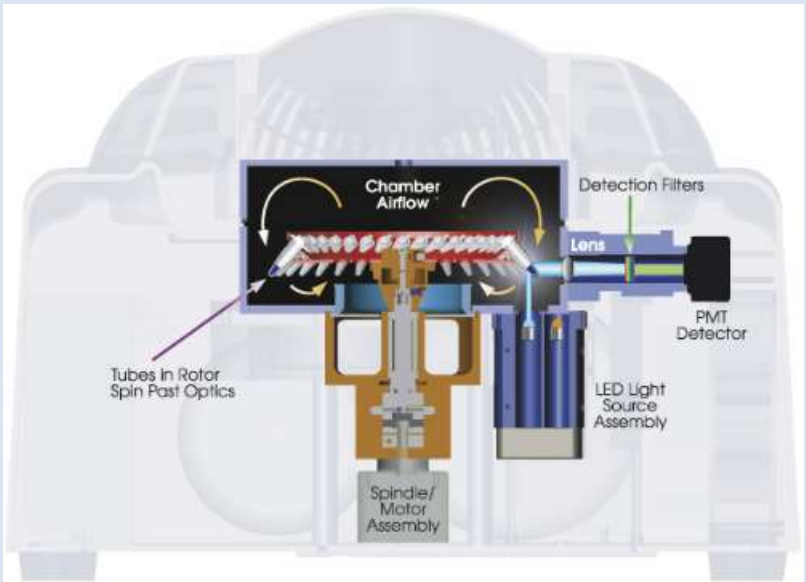


Figure 2.4. Cross-section of the Rotor-Gene™ 6000 real-time analyser showing the optical system. Heating/cooling is achieved by rapid airflow in the reaction chamber. Tubes spin past the excitation/detection optics every 150 milliseconds enabling high-speed data capture. Available from URL: http://www.qiagen.com/products/rotor-geneq.aspx#Tabs=t1. Accessed 28th August 2011.

Centrifugation prevents condensation and removes air bubbles but does not pellet DNA. Samples are heated and cooled in a low-mass air oven with heating achieved by a nickel-chrome element in the lid. The chamber is cooled by venting the air out through the top of the chamber while ambient air is blown up through the base. The Rotor-Gene™ 6000 series real-time analyser provides a choice of up to six excitation sources and six detection filters (Table 2.2) combined with a short fixed optical path to ensure consistent excitation as each sample is rotated around the reaction chamber eliminating the need for a passive reference dye.

Table 2.2. The excitation and detection ranges for the Rotor-Gene™ 6000 series real-time analyser. Six channels are available for optical detection on the Rotor-Gene 6000 Instrument. Available from URL: http://www.qiagen.com/products/rotor-geneq.aspx#Tabs=t1. Accessed 28th August 2011.

Channel	Excitation (nm)	Detection (nm)	Examples of fluorophores detected
Blue	365±20	460±20	Marina Blue®
Green	470±10	510±5	FAM®, SYBR® Green I
Yellow	530±5	557±5	JOE®, VIC®, HEX®, TET®
Orange	585±5	610±5	ROX®
Red	625±5	660±10	Cy5, LightCycler® Red640
Crimson	680±5	712 high pass	LightCycler® Red705

Samples are excited from the bottom of the chamber by a high-energy light-emitting diode (LED). Energy is transmitted through the thin walls at the base of the tube. Emitted fluorescence passes through emission filters on the side of the chamber and is collected by a photomultiplier. The Rotor-Gene™ 6000 series real-time analyser supports multiple tube formats by simply switching the metal rotor that holds the tubes. The 36-well rotor and locking ring for use with 0.2 ml reaction tubes and the 72-well rotor and locking ring for use with 0.1 ml strip reaction tubes (Corbett Research Ltd, Cambridge, UK) were used in all real-time thermal cycling.

2.6.5.2. ABI Prism® 7700 Real-Time Sequence Detection System

The ABI Prism® 7700 Real-Time Sequence Detection System is a 96-well block based thermal cycling system. The instrument utilises a single 10 mW Argon Ion Laser system as a light source with an output wavelength of 488 nm and is equipped with a charge-coupled device (CCD) camera that records all light between 500 nm and 650 nm of the fluorescent spectrum. The emission filters are optimised for use with standard dye sets: FAM™/SYBR® Green I, VIC®/JOE™, NED™/TAMRA™, and ROX® fluorescent dyes. The instrument utilises a passive internal reference to which the reporter dye signal can be normalised during data

analysis. Normalisation is necessary to correct for forestalment fluctuations caused by changes in concentration or volume.

2.7.6. Reaction Mix Preparation for One- Step or Two-Step real-time RT-PCR reactions

Real-Time PCR with either RT and PCR in a single reaction (one-step) or in separate reactions (two-step) were performed. In one-step RT-PCR, RT and PCR took place in one buffer system, which provided the convenience of a single-tube preparation for RT and PCR amplification. Two-step RT-PCR was performed in two separate reactions. First, total RNA was reverse transcribed into cDNA, then the cDNA is amplified by PCR.

In all instances, the total volume of reaction mix required was calculated according to the number of reactions and 10% extra i.e. for 10 reactions prepare sufficient master mix for 11 reactions to provide excess volume for the loss that occurs during reagent transfers.

Since it is virtually impossible to eliminate genomic DNA from RNA preparations, a "no-reverse transcriptase" control was included in all RT reactions. The "no-reverse transcriptase" control contained all the components of the reaction including the RNA template except for the reverse transcriptase enzyme. Reverse transcription therefore cannot take place. The presence of product in a PCR reaction was indicative of DNA contamination in the RNA preparation. Similarly, a “No Template Control” (NTC) was included in all PCR reactions to confirm the absence of cross-contamination of reagents and equipment. The NTC contained all the components of the reaction with exception of the nucleic acid template. The nucleic acid template was substituted with nuclease-free water (Bioline Ltd, London, UK). In both cases, the introduction of new reagents and treatment of equipment and instrumentation with cleaning products designed for the removal of contaminating DNA was required prior to all future RT and PCR reactions to prevent the generation of false positive results.

2.6.6.1. Two-step reverse-transcription polymerase chain reaction

Reverse Transcription

All RT reactions were performed using the TaqMan® Reverse Transcription Reagents (Applied Biosystems, Warrington, UK). The RT reaction mix was prepared by combining all the non-enzymatic components followed by the enzymatic components (Table 2.3) in a 1.5 ml microcentrifuge tube (Alpha Laboratories, Hampshire, UK). After mixing, the tubes were

centrifuged briefly to remove droplets of working RT reaction mix from inside the lid. Next, 12.3 µl of working RT reaction mix was aliquoted into 0.2 ml reaction tubes (Alpha Laboratories, Hampshire, UK). Finally, 7.7 µl of total RNA template was added to each tube.

Table 2.3. Preparation of working Reverse Transcription (RT) Reaction Mix for 1x reaction.

The non-enzymatic components are shown in BLUE and enzymatic components are shown in GREY.

Reaction Component	Volume (µl) per sample	Final Concentration
10 X TaqMan RT Buffer	1.0	1X
25 mM MgCl2	4.4	5.5 mM
dNTP Mixture	4.0	500 µM per dNTP
Random Hexamers	1.0	2.5 µM
Nuclease Inhibitor	0.4	0.4 U/µl
Multiscribe RT (50 U/µl)	0.5	1.25 U/µl
TOTAL	**12.3**	**-**

The reaction tubes were loaded into the chamber of a GeneAmp 2400 Thermal Cycler (Perkin-Elmer, UK) that was programmed with the following cycling parameters: 25^{O}C for 10 minutes, 48 ^{O}C for 30 minutes and 95^{O}C for 5 minutes. After thermocycling, the cDNA was stored at 2-8^{o}C overnight or at -20^{o}C for prolonged storage.

TaqMan® Universal PCR Master Mix

The TaqMan® Universal PCR Master Mix without UNG AmpErase (Applied Biosystems, Warrington, UK) was used in the second step of the two-step RT-PCR protocol. The TaqMan® Universal PCR Master Mix without UNG AmpErase contained AmpliTaq Gold® DNA polymerase, dNTPs with dUTP, carboxy-X-rhodamine (ROX™) Passive Reference, and optimised buffer components. A working reaction mix was prepared by combining the TaqMan® Universal PCR Master Mix without UNG AmpErase with forward and reverse primers, TaqMan® probe, and nuclease-free water (Bioline Ltd, London, UK) (Table 2.4) in a 1.5 ml microcentrifuge tube.

Table 2.4. Preparation of working TaqMan Universal PCR Master Mix without UNG AmpErase for 1X reaction.

Reaction Component	Volume (µl) per sample	Final Concentration
2X TaqMan® Universal PCR Master Mix	12.5	1X
Forward Primer	Variable	Optimal
Reverse Primer	Variable	Optimal
Probe	Variable	Optimal
Nuclease-free water	Variable	-
Total	**20**	**-**

The tube was vortexed briefly to mix the contents and followed by a brief centrifugation step to remove droplets of working reaction mix from inside the lid and eliminate any air bubbles.

Next, 20 µl of the reaction mixture was transferred to each well of a MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems, Warrington, UK) before 5 µl of cDNA template was added. The plate was centrifuged briefly to spin down the contents and eliminate any air bubbles present after covering with MicroAmp® Optical Caps (Applied Biosystems, Warrington, UK). Finally, the MicroAmp® Optical 96-Well Reaction Plate was loaded into the ABI Prism® 7700 Real-Time Sequence Detection System that was programmed with the cycling parameters shown in Table 2.5.

Table 2.5. Thermal Cycling Parameters for the TaqMan Universal PCR Master Mix without UNG AmpErase on the ABI Prism®7700 Fast Real-Time Sequence Detection System.

System	Polymerase activation[±]	PCR	
	HOLD	Cycle (45 cycles)	
		Denature	Anneal/extend
Temperature (°C)	95	95	60
Time (mm:ss)	10:00	00:15	1:00

[±]The 10-minute, 95 °C step is required to activate the AmpliTaq Gold® enzyme

2.6.6.2. One-step reverse-transcription polymerase chain reaction

The SuperScript™ III Platinum® One-Step Quantitative RT-PCR (qRT-PCR) System (Invitrogen, Paisley, UK) is a one-step, quantitative real-time RT-PCR system for the detection of RNA. This system combines the high-temperature reverse-transcription capability of SuperScript™ III Reverse Transcriptase with the automatic hot-start PCR provided by Platinum® *Taq* DNA polymerase. Both cDNA synthesis and PCR were performed in a single tube on the Rotor-Gene™ 6000 series real-time analyser. The working reaction mix was prepared by combining the SuperScript™ III Platinum® One-Step qRT-PCR System with forward and reverse primers and TaqMan® or TaqMan® MGB™ probe at optimised concentrations with nuclease-free water (Table 2.6) in a 1.5 ml microcentrifuge tube.

Table 2.6. Preparation of working reaction mix using the SuperScript® III Platinum® One-Step Quantitative RT-PCR System for 1X reaction.

Reaction Component	Volume (µl) per sample	Final Concentration
SuperScript™ II RT/Platinum® *Taq* Mix	0.8	-
2X Reaction Mix	12.5	1X
50 mM Magnesium Sulfate (MgSO4)	variable	3-6 mM
Forward Primer	Variable	Optimal
Reverse Primer	Variable	Optimal
Probe	Variable	Optimal
Nuclease-free water	Variable	-
Total	**20**	**-**

After mixing, a brief centrifugation step was performed to remove droplets of reaction mix from inside the lid of the 1.5 ml microcentrifuge tube and eliminate any air bubbles. Finally, 20 µl of reaction mixture was transferred into 0.2 ml reaction tubes or 0.1 ml strip reaction tubes (Corbett Research Ltd, Cambridge, UK) before 5 µl of total RNA was added exclusive of the NTC. RT-PCR was performed on the Rotor Gene 6000 (Corbett Research Ltd, Cambridge, UK) real-time system using the thermal cycling parameters described in Table 2.7.

Table 2.7. Thermal Cycling Parameters for the SuperScript® III Platinum® One-Step Quantitative RT-PCR System on the Rotor Gene 6000 real-time system.

System	RT activation	RT inactivation/ polymerase activation	PCR	
	HOLD	HOLD	Cycle (45 cycles)	
			Denature	Anneal/extend
Temperature (°C)	50	95	95	60
Time (mm:ss)	30:00	2:00	00:15	1:00

2.6.6.3. Preparation of carrier RNA

All dilutions of RNA were prepared in carrier RNA in order to limit possible RNA degradation due to RNase activity and to enhance recovery when purifying small amounts of RNA. To prepare a 1 µg/µl stock solution of cRNA, 1350 µl of nuclease-free water (Bioline Ltd, London, UK) was added to 1350 µg of lyophilised carrier RNA (QIAGEN Ltd, West Sussex, UK). The rehydrated carrier RNA was mixed thoroughly by vortexing and centrifuged briefly at 8,000 rpm to collect the contents at the bottom of the tube before it was divided into conveniently sized aliquots of 50 µl. Carrier RNA was stored at -20°C. To prepare a working solution of cRNA, 10 µl of the 1 µg/µl cRNA stock solution was added to 10 ml of nuclease-free water (Bioline Ltd, London, UK) to obtain a working solution of 1 ng/µl of cRNA. The working solution of 1 ng/µl of cRNA was used to prepare internal or positive controls.

2.6.6.4. Optimisation of Primer and Probe Concentrations of Singleplex Reactions

Real-time PCR assays require optimisation in order that robust assays are developed that are not affected by normal variations in the target DNA, primer, or probe compositions. A robust assay is defined as an assay in which these 'normal' variations cause no effect on the crossing threshold (C_T) and have only a minimal effect on the observed amount of fluorescence. The important criteria for optimisation are specificity, sensitivity, efficiency

and reproducibility (Edwards and Logan, 2009). The same principles of optimisation apply to assays run on all real-time platforms.

Determining Optimal Primer Concentration

A primer optimisation matrix composed of various permutations of forward and reverse primer in combination with a fluorogenic probe at a constant concentration was performed to determine optimal primer concentrations. The purpose of this procedure was to determine the minimum primer concentrations (Table 2.8). A PCR reaction mix was prepared using the TaqMan® Universal PCR Master Mix without UNG AmpErase (Applied Biosystems, Warrington, UK) to run four replicates of each of the 16 conditions and NTC.

Table 2.8. A primer optimisation matrix composed of various permutations of forward and reverse primer used in combination with a fluorogenic probe at a constant concentration to determine optimal primer concentrations.

	Forward Primer [nM]			
Reverse Primer [nM]	**50**	**300**	**500**	**900**
50	50/50	50/300	50/500	50/900
300	300/50	300/300	300/500	300/900
500	500/50	500/300	500/500	500/900
900	900/50	900/300	900/500	900/900

Primer optimisation was performed in a final reaction volume of 25 µl containing 1X TaqMan® Universal PCR Master Mix (Applied Biosystems, Warrington, UK), forward and reverse primer concentrations titrated from 50 nM to 900 nM in combination with 200 nM of probe and nuclease-free water (Bioline Ltd, London, UK) to a volume of 20 µl. Finally, 5 µL of cDNA was added exclusive of the NTC. Instead, 5 µl of nuclease-free water (Bioline Ltd, London, UK) was added. Determination of the volume of each component required for the four replicate reactions was achieved using a template devised in Microsoft Excel (Table 2.9). Parameters in the template were set in order to establish the volume of each reaction component required for the designated number of replicates. Primer optimisation was performed using the ABI Prism® 7700 Sequence Detection System (Applied Biosystems, Warrington, UK) using the thermal cycling parameters described in Table 2.5. At the end of runs, the results were tabulated for ΔRn. The minimum forward and reverse primer concentrations that yielded the maximum ΔRn were selected.

Table 2.9. Template for calculating the total volume of working reaction mix required for four replicate reactions of each of the 16 conditions and no template control. All parameters in blue boxes were set in order to establish the volume of each reaction component required for the designated number of replicates.

Set parameters		
Number of replicates	**4**	
Reaction volume	**25**	µl
Template volume per rxn	**5**	µl
Probe [ ] stock	**10**	µM
Probe final [ ]	**200**	nM
Forward primer [ ] stock	**20**	µM
Reverse primer [ ] stock	**20**	µM

Primer Concentration [nM]	**NTC**	**50/50**	**50/300**	**50/500**	**50/900**	**300/50**	**300/300**	**300/500**	**300/900**
Reaction Mix	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Forward Primer	1.5	0.3	0.3	0.3	0.3	1.5	1.5	1.5	1.5
Reverse Primer	1.5	0.3	1.5	2.5	4.5	0.3	1.5	2.5	4.5
Probe	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Template	0.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Water	45.0	27.5	26.3	25.3	23.3	26.3	25.0	24.0	22.0
Total volume	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**

Primer Concentration [nM]	**NTC**	**500/50**	**500/300**	**500/500**	**500/900**	**900/50**	**900/300**	**900/500**	**900/900**
Reaction Mix	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Forward Primer	1.5	2.5	2.5	2.5	2.5	4.5	4.5	4.5	4.5
Reverse Primer	1.5	0.3	1.5	2.5	4.5	0.3	1.5	2.5	4.5
Probe	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Template	0.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Water	45.0	25.3	24.0	23.0	21.0	23.3	22.0	21.0	19.0
Total volume	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**

Determining Optimal Probe Concentration

A probe optimisation matrix composed of five permutations of probe concentration was performed to determine the minimum probe concentration giving the minimum C_T for each probe target (Table 2.10). A PCR reaction mix was prepared to run four replicates of each of the five conditions and NTC. Probe optimisation was performed in a final reaction volume of 25 µl containing 1 X TaqMan® Universal PCR Master Mix (Applied Biosystems, Warrington, UK). The forward and reverse primer concentrations determined by the primer optimisation were used in the reaction mix in combination with probe concentrations titrated at 50 nM intervals from 50 to 250 nM and nuclease-free water (Bioline Ltd, London, UK) to a volume of 20 µl. Finally, 5 µl of cDNA was added exclusive of the NTC. Instead, 5 µl of nuclease-free water was added (Bioline Ltd, London, UK).

Table 2.10. A probe optimisation matrix composed of five permutations of probe performed in combination with optimised forward and reverse primer concentrations to determine the optimal probe concentration.

	Forward Probe [nM]				
Reverse Probe [nM]	**50**	**100**	**150**	**200**	**250**
50	50/50	50/100	50/150	50/200	50/250
100	100/50	100/100	100/150	100/200	100/250
150	150/50	150/100	150/150	150/200	150/250
200	200/50	200/100	200/150	200/200	200/250
250	250/50	250/100	250/150	250/200	250/250

Determination of the volume of each component required for the four replicate reactions was achieved using a template devised in Microsoft Excel (Table 2.11). Parameters in the template were set in order to establish the volume of each reaction component required for the designated number of replicates. The probe optimisation was performed using the ABI Prism® 7700 Sequence Detection System (Applied Biosystems, Warrington, UK) using the thermal cycling parameters described in Table 2.5. At the end of runs, the results were tabulated for C_T. The minimum probe concentration that yielded the minimum C_T was selected.

Table 2.11. Template for calculating the total volume of working reaction mix required for four replicate reactions of each ofthe five conditions.

All parameters in blue boxes were set in order to establish the volume of each reaction component required for the designated number of replicates.

Set parameters		
Number of replicates	**4**	
Reaction volume	**25**	µl
Template volume per rxn	**5**	µl
Probe [ ] stock	**10**	µM
Forward primer [ ] stock	**20**	µM
Reverse primer [ ] stock	**20**	µM

Primer	**Final [nM]**
Forward	**500**
Reverse	**300**

Probe Concentration [nM]	**NTC**	**50**	**100**	**150**	**200**	**250**
Reaction Mix	50	50	50	50	50	50
Forward Primer	2.5	2.5	2.5	2.5	2.5	2.5
Reverse Primer	1.5	1.5	1.5	1.5	1.5	1.5
Probe	1.0	0.5	1.0	1.5	2.0	2.5
Template	0	20	20	20	20	20
Water	45	25.5	25	24.5	24	23.5
Total volume	**100**	**100**	**100**	**100**	**100**	**100**

The SuperScript™ III Platinum® One-Step qRT-PCR System (Invitrogen, Paisley, UK) (Section 2.6.6.2) was used as an alternative to the TaqMan® Universal PCR Master Mix (Applied Biosystems, Warrington, UK) for determining optimal primer and probe concentrations. A working reaction mix was prepared by combining the SuperScript™ III Platinum® One-Step qRT-PCR System with the same permutations of forward and reverse primer and TaqMan® probe as described in Table 2.8 and Table 2.10, respectively. Primer and probe optimisation using the SuperScript™ III Platinum® One-Step qRT-PCR System was performed on the Rotor-Gene™ 6000 series real-time analyser (Corbett Research Ltd, Cambridge, UK) using the thermal cycling parameters outlined in section 2.6.6.2.

2.7.7. Purification of amplification products

Amplification products were purified using the QIAquick® PCR purification kit (QIAGEN Ltd, Crawley, West Sussex, UK). This commercial purification kit facilitated the removal of excess nucleotides and enzyme contamination from DNA fragments that would otherwise interfere with subsequent downstream applications using the selective binding properties of a silica membrane combined with spin-column technology. The protocol was performed according to manufacturer's instructions. Briefly, five volumes of binding buffer PB containing pH

indicator I was added to 1 volume of the PCR sample and mixed in a 1.5 ml microcentrifuge tube (Alpha Laboratories, Hampshire, UK). The colour of the mixture turned to yellow. However, if the colour of the mixture was orange or violet, 10 µl of 3 M sodium acetate, pH 5.0 (Sigma-Aldrich, Dorset, UK) was added to correct the pH of the binding mixture before proceeding with the protocol. A QIAquick spin column was placed in a 2 ml collection tube. To bind DNA, the sample was applied to the QIAquick column by pipetting. The column was centrifuged for 60 seconds at 13,000 rpm. The flow-through was discarded. The QIAquick spin column was placed back into the same collection tube and 0.75 ml of ethanol-containing Buffer PE was added by pipetting. The spin column was centrifuged at 13,000 rpm for 60 seconds to wash away unwanted primers and impurities, such as salts, enzymes, and unincorporated nucleotides. The flow-through was discarded and the QIAquick spin column was placed back into the same collection tube. The column was centrifuged for an additional 60 seconds at 13,000 rpm to remove any residual Buffer PE that may interfere with subsequent enzymatic reactions. The QIAquick spin column was placed into a clean 1.5 ml microcentrifuge tube (Alpha Laboratories, Hampshire, UK). To elute DNA, 30 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) was added to the centre of the QIAquick membrane for complete elution of bound DNA. The column was incubated at room temperature (15°C to 25°C) for 60 seconds. The column was centrifuged for 60 seconds at 13,000 rpm. The purified DNA was stored at –20°C until required. The purified product was run on a 2% agarose gel as described in section 3.3.15 in order to assess the quality and quantity of DNA.

CHAPTER THREE

3. Molecular Detection of a Novel Respiratory Virus

3.1. Introduction

A longitudinal study conducted using prospectively acquired respiratory samples collected over a period of 25 years from otherwise healthy children with LRTI offered significant advancement in our knowledge of HMPV (Williams *et al*., 2004). Along with accumulating evidence from Europe, North America, and Australia, this careful study indicated that HMPV is, with a few differences, a kid brother or sister to HRSV that, particularly in young children, accounts for a very substantial proportion of cases previously relegated to the "undiagnosed" category (McIntosh and McAdam, 2004). Indeed, this substantial proportion accounted for 20% of all previously virus-negative lower respiratory tract illnesses (Williams *et al*., 2004). These findings made the implementation of tools to allow detection of this emerging virus an important priority.

The introduction of nucleic acid amplification techniques has largely surmounted the problems that thwarted the diagnosis of HMPV within routine diagnostic laboratories (Galiano *et al*., 2004; Kuypers *et al*., 2005; Mackay *et al*., 2003; Hopkins *et al*., 2008). While these techniques seem to present the perfect answer to the dilemmas associated with the diagnosis of respiratory infection attributed to HMPV, limitations do exist within this technology. Indeed, an inherent problem in diagnostic nucleic acid amplification techniques is the presence of amplification inhibitors (Dreier *et al*., 2005). Numerous substances have the potential to inhibit this exquisitely sensitive methodology (Al-Soud *et al*., 2000; 2001; Khan *et al*., 1991; Lantz *et al*., 1997; Hartman *et al*., 2005; Monteiro *et al*., 1997; Satsangi *et al*., 1994; Wang *et al*., 1992). The presence of inhibitors has the potential to increase error, reduce assay resolution, and produce false results in both quantitative and qualitative molecular tests (Huggett *et al*., 2008). The extraction and partial purification of high quality nucleic acid, DNA or RNA, from clinical specimens is a critical first step in the successful amplification and detection of the genome of any pathogen that may be present (Clewley, 1999). Successful isolation of nucleic acids from clinical specimens can improve the reproducible performance of downstream applications even with specimens containing very low amounts of nucleic acid (Alp and Hascelik, 2009). If, however, the nucleic acid is lost during the extraction process, broken down, or contains compounds that inhibit the reverse transcriptase or *Taq* polymerase enzymes used in PCR, then the whole procedure will fail, yielding a falsely negative result. Similarly, if during extraction the nucleic acid becomes cross-contaminated with genomic fragments from other specimens containing the same

pathogen, or with previous amplified PCR products, a falsely positive result will be obtained (Clewley, 1999). The procedure is complicated further by the fact that, chemically and biologically, RNA is significantly more labile that DNA (Promega, 1997). This feature coupled with the short half life of RNA once extracted and susceptibility to degradation by ubiquitous RNases make effective and reproducible purification of RNA from various heterogeneous materials a challenge (Chan and Yiap, 2009; Vomelová *et al*., 2009). Nevertheless, nucleic acid extraction is the basis for all the subsequent steps of genomic detection and characterisation even though it can be sometimes technically demanding (Clewley, 1999).

The incorporation of a universal exogenous internal control into PCR reactions has become an important strategy to monitor the combined effect of extraction and amplification, address the problems associated with the presence of PCR inhibitors, and raise confidence in results generated for the viral target of interest. There are two main strategies for the use of an exogenous internal control (Hoorfar *et al*., 2004). A competitive or homologous internal control uses the same primer pair as the viral target sequence, but with an altered probe binding sequence (Stevenson *et al*., 2008). These controls most closely mimic amplification of the target sequence, but can potentially compete with target amplification to compromise the overall detection limit of the assay, especially if the target microbe is present at low levels (Dingle *et al*., 2004; Stevenson *et al*., 2008). Another limitation is that they normally require a different internal control for each assay and are therefore incompatible with multiplex PCR in which several primer pairs are required (Dingle *et al*., 2004; Stevenson *et al*., 2008). Non-competitive or heterologous internal controls consist of separate amplifiable targets. Since these do not contain the target sequence, a separate primer pair and probe are required to amplify the internal control and the target (Espy *et al*., 2006). Such controls include house-keeping genes that occur naturally in all human nucleated cell types (Espy *et al*., 2006) and armoured RNA (Beld *et al*., 2004; Eisler *et al*., 2004; Huang *et al*., 2008; Meng and Li., 2010; Stevenson *et al*., 2008). Animal viruses are an attractive and versatile alternative for use as DNA or RNA internal controls since the assumption is that intact virus, when used as a universal internal control, behaves more similarly in the extraction procedure to the target virus of interest, in contrast to using, for example, a plasmid as an internal control (Niesters, 2004). Animal viruses used as universal DNA and RNA internal controls include bovine viral diarrhoea virus (BVDV) (Cleland *et al*., 1999), feline calicivirus (FCV) (Mattison *et al*., 2009), mengovirus (Comelli *et al*., 2008), PDV (Clancy *et al*., 2008), phocine herpes virus type 1 (PhHV-1) (van Doornum *et al*., 2003), murine cytomegalovirus (mCMV) (Garson *et al*., 2005). As established model viruses that

serve as surrogate markers for human disease and/or viruses that either closely resemble a human virus or belong to the same family as a human counterpart, animal viruses present good candidates for internal controls (Bidawid *et al*., 2003; Costafreda *et al*., 2006; Wilson *et al*, 2008; Yoo *et al*., 2006). Furthermore, these animal viruses grow readily in virus culture and produce clear cytopathic effects in cultures of established cell lines (Bidawid *et al*., 2003) making them easily accessible for use in clinical virology setting for routine molecular diagnosis. A newer alternative that is becoming increasingly popular is the use of RNA and DNA bacteriophages as internal controls (Blaise-Boisseau *et al*., 2010; Dreier *et al*., 2005; Gerriets *et al*., 2008; Ninove *et al*., 2011; Rolfe *et al*., 2007). The *Escherichia coli* bacteriophage MS2 has proved an ideal candidate for use as an internal control in RT-PCR assays (Blaise-Boisseau *et al*., 2010; Dreier *et al*, 2005; Ellis and Curran, 2010; Ninove *et al*., 2011; Rolfe *et al*., 2007). It is prone to the same inhibition/degradation as RNA viruses, is non-infectious, stable, and easily propagated (Rolfe *et al*., 2007).

3.2. Aims

The premise of this initial venture was the introduction of a molecular diagnostic test for the detection of a newly discovered virus, HMPV. However, the basis for this introduction was not only the selection of an appropriate nucleic acid detection system. It was envisaged that this test would become part of the routine diagnostic testing repertoire for respiratory viruses and therefore a robust approach was required. This necessitated the incorporation of an internal control and the development of detection system to allow the measurement of multiple targets within a single tube format as the internal control would co-amplify with the target of interest. However, the most labour-intensive and critical step remaining for consideration was the efficient extraction of nucleic acids from different clinical samples (Niesters, 2004). Nucleic acid extraction presents a potential bottleneck to the successful implementation of nucleic acid detection within a routine virological setting (Niesters, 2004) and so the introduction of a method to allow the automation of this process was essential.

3.3. Materials and Methods

3.3.1. Positive control material

HMPV positive culture supernatant kindly donated by Dr Terry Collins, Specialist Virology Centre, Gartnavel General Hospital, Glasgow, Scotland facilitated optimisation of the selected monoplex real-time RT-PCR assay in the absence of a commercial working reagent for HMPV and preceding identification of positive samples by the monoplex real-time RT-

PCR assay. Further HMPV positive culture supernatant was identified through evaluation of the monoplex real-time RT-PCR. Influenza viruses were identified by routine examination of respiratory specimens by virus culture, and DFA (IMAGEN™, Oxoid Ltd, Hampshire, UK) (Section 2.4.2).

3.3.2. Characteristics of patients and specimens tested

In total, 128 respiratory specimens were submitted to the Microbiology Department between September 2007 and April 2008 for routine examination by virus culture, DFA (IMAGEN™, Oxoid Ltd, Hampshire, UK) for influenza virus types A and B, HRSV, PIV types 1-3, HRSV, adenovirus, and *Chlamydia* sp., and NOW® RSV Test (Binax, Inc., ME, USA) (Section 2.4.2). A frozen aliquot of each sample was stored at -70°C for retrospective evaluation of the COBAS® AmpliPrep TNAI Kit on the COBAS® AmpliPrep Instrument. Clinical specimens were predominantly from children ≤ 18 years (n= 103; 80.5% NPA samples) that attended the Norfolk and Norwich University Hospital with symptoms of acute respiratory illness. Other specimen types included 7 combined nose and throat swabs, 16 throat swabs, 1 sputum specimen, and 1 BAL specimen. Six combined nose and throat swabs and 9 throat swabs were collected from patients of all ages that presented with ILI or other acute respiratory illness to sentinel general practices that participated in the national virological influenza surveillance scheme during winter 2007/2008.

3.3.3. Primer and probe design

3.3.3.1. Human metapneumovirus

The primer and probe sequences selected for detection of HMPV by real-time RT-PCR were designed according to Maertzdorf *et al*. (2004) (Table 3.1). The probe was labelled at the 5' end with 6-carboxyfluorescein (6-FAM) and at the 3' end with the non-fluorescent quencher Black Hole Quencher™ 1 (BHQ-1).

Table 3.1. Primer and probe sequences for the detection of human metapneumovirus.
The probe was labelled at the 5' end with 6-carboxyfluorescein (6-FAM) and at the 3' end with a non-fluorescent quencher, Black Hole Quencher™ 1 (BHQ-1). Residue Y in the third position of the NL-N probe represents either a cysteine (C) or a thymidine (T) residue.

Primer or probe	Sequence (5'-3')	Target Gene
NL-N forward	CAT ATA AGC ATG CTA TAT TAA AAG AGT CTC	Nucleoprotein
NL-N reverse	CCT ATT TCT GCA GCA TAT TTG TAA TCA G	
NL-N probe	6-FAM – TGY AAT GAT GAG GGT GTC ACT GCG GTT G – BHQ-1	

The primer and probe sequences were located within the most conserved region of the N protein gene of the virus (Maertzdorf *et al*., 2004). This region was identified by the creation of entropy plots of oligonucleotide-annealing sites within the HMPV genome with the BioEdit software package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Entropy plots also facilitated the identification of potential mismatches of the primer and probe sequences with the respective target sequence. Entropy plots of oligonucleotide-annealing sites in four different primer and probe sets of the NL-N, ALT-N, N, and L gene assays were compared (Figure 3.1).

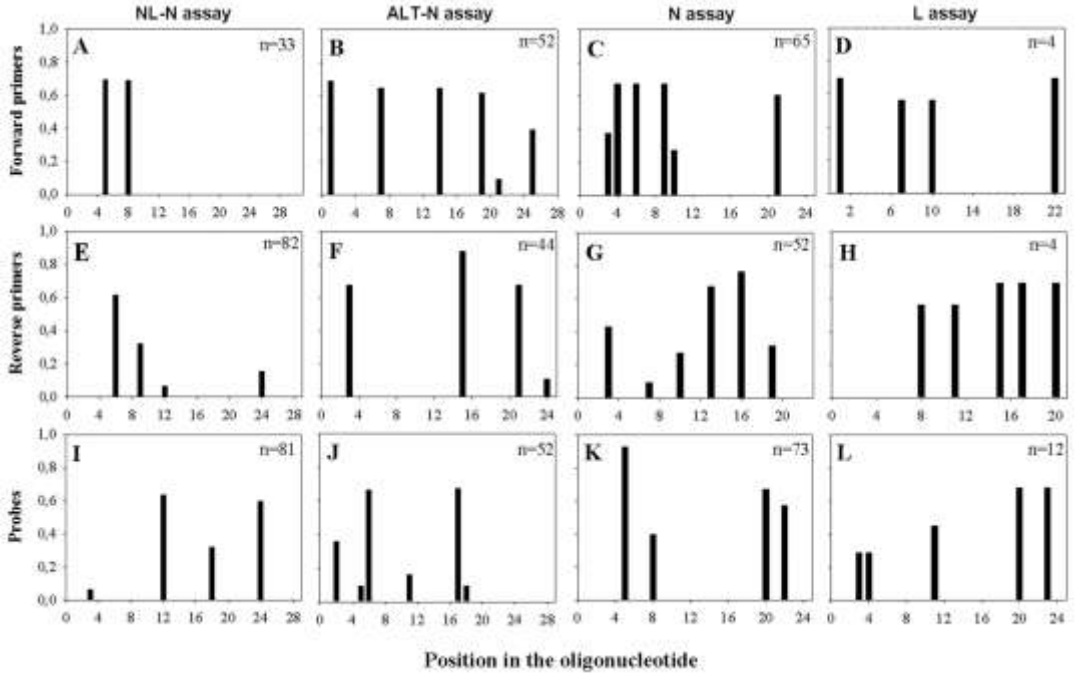


Figure 3.1. Entropy plots of oligonucleotide-annealing sites in four different primer and probe sets showing the mismatches of each of the primer and probe sequences with the target sequences. (Maertzdorf *et al*., 2004).

The N and L gene assays were previously described by Mackay *et al*., (2003), and van den Hoogen *et al*., (2003) while the NL-N and ALT-N assays were newly designed by Maertzdorf *et al*., (2004). A good match of the 3' end of the primer set and 5' end of the probe is critical to successful detection of viral RNA and to minimise the risk of false-negative results (Maertzdorf *et al*., 2004). Entropy plots show the sequences recognised by the individual oligonucleotides in each primer and the probe set when compared to all available nucleotide sequences of HMPV. The heterogeneities were displayed as entropy values on the *x*-axis. A higher entropy value indicated mismatches at a particular position in the oligonucleotide with a larger number of target sequences analysed. The number of sequences upon which

each plot was based was given in the upper right corner of each plot. Oligonucleotide positions were given in the 5'-3' direction, with position 1 being the extreme 5' nucleotide. The primer and probe sequences of the NL-N gene assay contain the least number of mismatches compared to the target sequences of all the assays that were compared.

3.3.3.2. Influenza viruses

The primer and probe sequences selected for detection of influenza virus types A and B were taken from NSM VSOP 25 entitled "*Real-Time Quadriplex PCR for the Detection of Influenza*" (HPA, 2006) issued by the Standards Unit, DEST, Cfl, HPA (Appendix III). The real-time RT-PCR assay incorporated primers and TaqMan® or TaqMan® MGB™ probes for the detection of all generic influenza virus A subtypes (H1-H15) and influenza virus B. The influenza virus A and influenza virus B oligonucleotides were designed from multiple alignments of matrix and nucleoprotein gene sequences and designed to target a 205 base pair (bp) region and a 148 bp region, respectively. Primers and TaqMan® MGB™ probe targeted a 151 bp region in the haemagglutinin gene of influenza A H5 viruses only to distinguish Influenza A H5 viruses from other influenza viruses. MS2 bacteriophage was incorporated as an internal control and specific primers and probe were designed to target a 99 bp region near the 5' end of the genome. No modifications were made to the original primer and probe sequences described. The sensitivity of the real-time quadriplex PCR for the detection of influenza was determined using titrated stocks of influenza virus A and influenza virus B (HPA, 2006). The specificity of the real-time quadriplex PCR for the detection of influenza was determined using all 15 subtypes of influenza virus type A (H1-H15), the full range of influenza H5 viruses, including influenza A H5 viruses isolated in Vietnam in 2004 and Turkey in 2005, and a blind respiratory panel consisting of influenza A virus subtypes H1, H3 and, H5, influenza virus B, and other respiratory pathogens (HPA, 2006).

3.3.3.3. MS2 Bacteriophage

The primer and probe sequences selected for detection of MS2 Bacteriophage were taken from NSM VSOP 25 entitled "*Real-Time Quadriplex PCR for the Detection of Influenza*" (HPA, 2006) issued by the Standards Unit, DEST, Cfl, HPA (Appendix III). No modifications were made to the original primer and TaqMan® probe sequences.

3.3.3.4. Onderstepoort strain of canine distemper virus

Sequence data for the Onderstepoort strain of canine distemper virus was obtained from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov, accession number AF378705). Conserved regions within the genome of Onderstepoort strain of canine distemper virus were identified by generating multiple sequence alignments using the BioEdit software package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The N gene was selected for development of novel primer and probe sequences due to the high level of sequence conservation within this region of the genome. The nucleotide sequence of the N gene is a 1.57 kilobase (kb) region that stretches from base 108 to 1679. Primer and probe sequences were designed using the Primer Express Software (Applied Biosystems™, Warrington, UK) to amplify a 77 bp fragment within N gene of the virus. The selected regions of the genome covered by the forward primer, reverse primer and probe were nucleotide positions 1216 to 1235, 1269 to 1292, and 1238 to 1259 respectively (Figure 3.2). The probe was labelled at the 5' end with the fluorophore ROX and at the 3' end with the non-fluorescent quencher, Black Hole Quencher™ 2 (BHQ-2) (Table 3.2).

Table 3.2. Primer and probe sequences for the detection of the Onderstepoort strain of canine distemper virus.

The probe was labelled at the 5' end with ROX (carboxy-X-rhodamine) and a non-fluorescent quencher, Black Hole Quencher™ 2 (BHQ-2) at the 3' end.

Primer or probe	Sequence (5'-3')	Target gene
Forward primer	GGA GAT CTG CCG GCA AAG TA	Nucleoprotein
Reverse primer	TGA CAC TAG CTG AGC TTC CTC CTT	
Probe	ROX – CTC TGC ACT TGC CGC CGA GCT T – BHQ-2	

The degree of nucleotide sequence homology was checked using the BLAST algorithm available from the NCBI that provides sequence similarity searches of databases maintained by the NCBI, EMBL, and DDBJ.

ATGGCTAGCCTTCTTAAAAGCCTCACACTGTTCAAGAGGACTCGGGACCAACCCCCTCTTGCCTCTGGCT

CCGGGGGAGCAATAAGAGGAATAAAGCATGTCATTATAGTCCTAATCCCGGGTGATTCAAGCATTGTTAC

AAGATCTCGACTATTGGATAGACTTGTTAGGTTGGTTGGTGATCCAAAAATCAACGGCCCTAAATTAACT

GGGATCTTAATCAGTATCCTCTCCTTGTTTGTGGAATCCCCTGGACAGTTGATCCAGAGGATCATAGACN

ACCCTGATGTAAGCATCAAGTTAGTAGAGGTAATACCAAGCATCAACTCTGTTTGCGGTCTTACATTTGC

ATCCAGAGGAGCAAGTCTGGATTCTGAGGCAGATGAGTTCTTCAAAATTGTAGACGAAGGGTCGAAAGCT

CAAGGGCAATTAGGCTGGTTAGAGAATAAGGATATAGTAGACATAGAAGTTGATAATGCTGAGCAATTCA

ATATATTGCTAGCTTCCATCTTGGCTCAAATTTGGATCCTGCTAGCTAAAGCGGTGACTGCTCCTGATAC

TGCAGCCGACTCGGAGATGAGAAGGTGGATTAAGTATACCCAGCAAAGACGTGTGGTCGGAGAATTTAGA

ATGAACAAAATCTGGCTTGATATTGTTAGAAACAGGATTGCTGAGGACCTATCTTTGAGGCGATTCATGG

TGGCACTCATCTTGGACATCAAACGATCCCCAGGGAACAAGCCTAGAATTGCTGAAATGATTTGTGATAT

AGATAACTACATTGTGGAAGCTGGGTTAGCTAGTTTCATCCTAACTATCAAGTTTGGCATTGAAACTATG

TATCCGGCTCTTGGGTTGCATGAGTTTTCCGGAGAATTAACAACTATTGAATCCCTCATGATGCTATATC

AACAGATGGGTGAAACAGCACCGTACATGGTTATCTTGGAAAACTCTGTTCAAAACAAATTTAGTGCAGG

GTCCTACCCATTGCTCTGGAGTTATGCTATGGGGGTTGGTGTTGAACTTGAAAACTCCATGGGAGGGTTA

AATTTCGAGTCGTCTTACTTTGACCCAGCTTACTTCAGACTCGGGCAAGAAATGGTTAGGAGATCTGCCG

GCAAAGTAAGCTCTGCACTTGCCGCCGAGCTTGGCATCACCAAGGAGGAAGCTCAGCTAGTGTCAGAAAT

AGCATCCAAGACAACAGAGGACCGGACAATTCGAGCTACTGGTCCTAAGCAATCCCAAATCACTTTTCTG

CACTCGGAAAGATCCGAAGTCGCCAATCAACAACCCCCAACCATCAACAAGAGGTCCGAAAACCAGGGAG

GAGACAAATACCCCATTCACTTCAGTGACGAAAGGCTTCCAGGGTATACCCCAGATGTCAACAGTTCTGA

ATGGAGTGAGTCACGCTATGACACCCAAATTATCCAAGATGATGGAAATGACGATGACCGGAAATCGATG

GAAGCAATCGCCAAGATGAGGATGCTTACTAAGATGCTCAGTCAACCTGGGACCAGTGAAGATAGTTCTC

CTGTTTATAATGATAGAGAGCTACTCAATTAA

Figure 3.2. Target oligonucleotide sequences in the nucleocapsid protein gene of the Onderstepoort strain of canine distemper virus (accession number AF378705).

The nucleocapsid (N) protein gene sequence data was obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov. The nucleotide sequence of the N protein gene is a 1.57 kb region from base 108 to 1679. The forward primer, reverse primer, and probe represented in green, blue, and red, respectively, target a 77 bp region within the N protein gene of the virus.

3.3.4. Detection of influenza viruses using the real-time quadriplex RT-PCR assay

A working reaction mix was prepared according to NSM VSOP 25 entitled "*Real-Time Quadriplex PCR for the Detection of Influenza*" (HPA, 2006) by combining the SuperScript™ III Platinum® One-Step qRT-PCR System (Invitrogen Ltd, Paisley, UK) with primers and TaqMan® probes or TaqMan® MGB™ probes at the concentrations shown in Table 3.3 with nuclease-free water (Bioline Ltd, London, UK) to a volume of 20 µl. Finally, 5 µl of viral RNA extract was added exclusive of the NTC. Instead, 5 µl of nuclease-water was added. RT-PCR was performed on the Rotor-Gene 6000 real-time system (Corbett Research Ltd, Cambridge, UK) using the thermal cycling parameters (Section 2.6.6.2) acquiring on the FAM, VIC, CY-5 and ROX channels. Real-time measurements were taken at each cycle.

Table 3.3. Preparation of working reaction mix for the real-time quadriplex RT-PCR assay.
The volume of each reaction component was taken from National Standard Method VSOP 25 entitled "*Real-Time Quadriplex PCR for the Detection of Influenza*" issued by the Standards Unit, Department for Evaluations, Standards and Training, Centre for Infections, Health Protection Agency (HPA, 2006).

Reagents	**Volume (µl) per 25 µl reaction**	**Stock Concentration**	**Final Concentration**
SuperScript III RT/Platinum® *Taq* Mix	12.5	-	-
2X Reaction Mix	0.80	2X	1X
H5 forward primer	1.00	10 µM	400 nM
H5 reverse primer	1.00	10 µM	400 nM
H5 probe	0.50	5 µM	100 nM
FA forward primer	0.50	20 µM	400 nM
FA reverse primer	1.00	20 µM	800 nM
FA forward primer	0.40	10 µM	160 nM
FB forward primer	0.17	20 µM	132 nM
FB reverse primer	0.17	20 µM	132 nM
FB probe	0.20	3.3 µM	26 nM
MS2 forward primer	0.10	20 µM	80 nM
MS2 reverse primer	0.10	20 µM	80 nM
MS2 probe	0.20	10 µM	80 nM
Nuclease-free water	1.37	-	-
TOTAL VOLUME	20.00		

Abbreviations: H5, avian influenza virus H5N1; FA, influenza virus type A; FB, influenza virus type B; MS2, MS2 Bacteriophage.

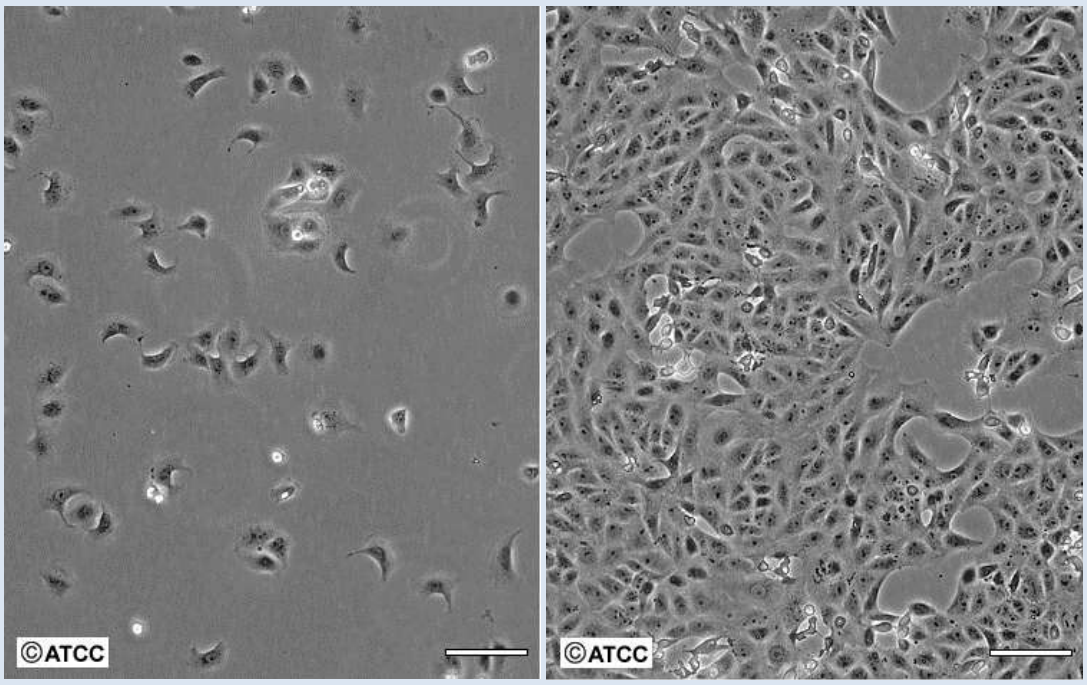
3.3.5. Growth of the Onderstepoort strain of Canine Distemper Virus

3.3.5.1. EMEM maintenance medium

This complete medium was supplied ready for use in 100 ml volumes with serum by the CMPHL, Addenbrooke's Hospital, Cambridge. It was prepared from a basal medium of Eagle's minimal essential medium (EMEM) containing sodium bicarbonate, L-glutamine, and pH indicator and supplemented with 50 IU/ml penicillin, 100 µg/mL streptomycin, 2.5 µg/mL amphotericin and 1% Foetal Calf Serum (FCS). The addition of phenol red to the EMEM served to indicate changes in pH. EMEM with 1% FCS was stored at 2°C to 8°C.

3.3.5.2. Cell culture monolayers

Cell culture monolayers of kidney cells of a normal African green monkey (Vero) (Figure 3.3) were supplied ready to use in disposable glass tubes by the CMPHL, Addenbrooke's Hospital, Cambridge. On receipt, the tubes were placed in a rack at an angle of approximately 5° and incubated overnight at 37°C. On the following day, the cells were examined microscopically using an inverted microscope to assess cell growth. Cells that had formed an approximately 80% confluent monolayer were incubated 35°C until required. Fresh medium was applied every 3-4 days to maintain the condition of the cells.



Low Density Scale Bar = 100 µM High Density Scale Bar = 100 µM

Figure 3.3. Cell micrographs of uninfected low and high density Vero cells derived from kidney cells of a normal African green monkey (*Cercopithecus aethiops*).

Images are available from URL: http://www.lgcstandards-atcc.org. Accessed 5th December 2010.

3.3.5.3. Onderstepoort strain of canine distemper virus

Cell culture supernatant containing the Onderstepoort strain of canine distemper virus was kindly donated by Professor Louise Crosby, Queen's University Belfast, Northern Ireland.

3.3.5.4. Specimen Inoculation for virus cell culture

The Onderstepoort strain of canine distemper virus was inoculated onto confluent continuous cell culture monolayers of Vero cells (Figure 3.3). A sterile pastette was used to transfer 6 drops (0.2 ml) of well-mixed cell culture supernatant to the selected cell line. The tubes were incubated overnight at 37^{o}C in a rack at an angle of approximately 5^{o} to allow the inocula to absorb to the cell monolayer. The cultures were then re-fed with 1 ml of fresh EMEM maintenance medium and incubated at 37^{o}C on a roller drum for no longer than 3 days to maintain a low multiplicity of infection (MOI). The tubes were observed every 24 or 48 hours to check for the development of cytopathic effects and contamination under light microscopy. Typical CPE characteristics observed for the Onderstepoort strain of canine distemper virus growing in continuous cell culture monolayers of Vero cells included cell-rounding, detachment of cells and the destruction of the cell monolayer. Next, the cells were scraped into the medium. The tubes were vortexed briefly and then centrifuged at 3000 rpm for 5 minutes to remove the cellular debris. The supernatant was harvested and 6 drops (0.2 ml) of supernatant was passaged onto fresh Vero cell monolayers. The remaining supernatant was stored at -70°C until required.

3.3.6. Propagation of *Escherichia coli* Phage MS2

The production of a high titre suspension of MS2 bacteriophage was achieved using the Double Agar Overlay method otherwise known as the "soft agar overlay", "double agar layer" or "double layer" method of plaque assay (Kropinski *et al*., 2009), which was kindly donated by Dr Martin Curran, CMPHL, Addenbrooke's Hospital, Cambridge. Dilutions of phage suspension were mixed with host bacteria in a dilute, molten agar or agarose matrix – the "top agar" or "overlay", which was distributed evenly to solidify on a standard agar plate – the "bottom agar" or "underlay" (Kropinski *et al*., 2009). After incubation, the plaques were visualised as zones of clearing or diminished growth in the bacterial lawn, which grow in the overlay (Kropinski *et al*., 2009).

3.3.6.1. Stock Cultures

Escherichia coli Phage MS2 (15597-B1™) and its bacterial *E. coli* host (15597™) were acquired from the American Type Culture Collection (ATCC) (LGC standards, UK). The freeze-dried cultures were stored at 5^{o}C or colder until medium were prepared to initiate the revival of the freeze-dried material as directed by the instructional guide provided by the ATCC.

3.3.6.2. Re-hydration of freeze-dried *Escherichia coli* host and host propagation

The freeze-dried culture of *E. coli* was revived by adding 500 µl of #271 broth medium (Appendix II) to the freeze-dried material under aseptic conditions. The rehydrated culture was mixed well and then transferred to a 25 ml sterile universal containing 6 ml of #271 broth medium. The last remaining drops of the rehydrated culture were transferred to the prepared nutrient agar slant (Appendix II). The enrichment broth and nutrient agar slant were incubated aerobically at 37°C for 24 hours. Following incubation, the enrichment broth was subcultured onto a solid agar plate prepared from #271 broth medium (Appendix II). The inoculum was dispersed over the surface of the agar plate by the surface streak method in order to achieve the isolation of individual bacterial colonies (Figure 3.4). The agar plate was incubated aerobically at 37°C for 18-24 hours.

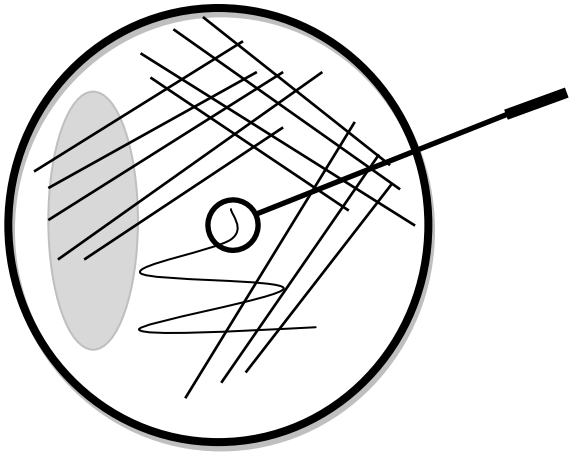


Figure 3.4. Schematic representation of the surface streak method used to disperse the inoculum over the surface of each agar plate in order to isolate individual bacterial colonies.
Adapted from the National Standard Method Virology Standard Operating Procedure 52 entitled "*Inoculation of Culture Media*" issued by the Standards Unit, Department for Evaluations, Standards and Training, Centre For Infections, Health Protection Agency (HPA, 2008).

3.3.6.3. Re-hydration of freeze-dried bacteriophage

To recover the *E. coli* Phage MS2 from the freeze-dried vial, the phage specimen was re-hydrated with 1 ml of #271 broth medium under aseptic conditions. The vial was mixed well and then 0.25 ml of the re-hydrate was used to prepare a new high titre phage suspension. The remaining phage mixture was preserved in a sterile screw-capped vial at -70^{o}C.

3.3.6.4. Preparation of broth culture of *Escherichia coli* host

It was essential that an actively growing broth culture of the *E. coli* host was prepared before opening the phage specimen. To prepare the actively growing broth culture of the host, a single isolated colony of *E. coli* was inoculated into 6 ml of #271 broth medium. The broth culture was incubated at 37°C for 18-24 hours prior to inoculation with revived phage.

3.3.6.5. Preparation of a high-titre phage suspension

A high-titre phage suspension was prepared by adding 200 µl of *E. coli* host and 50 µl of MS2 bacteriophage to a sterile universal. This process was repeated 5 times. The suspension was incubated at room temperature (15°C to 25°C) for 10 minutes to allow the phage to adsorb onto the host. A control universal containing only the *E. coli* host was prepared by adding 200 µl of *E. coli* to 50 µl of #271 broth medium. Next, 100 ml of soft overlay agar (Appendix II) was melted in a microwave oven. The molten agar was cooled to approximately 50^{0}C before aseptically adding 1 ml of 10% glucose (Appendix II), 0.2 ml of 1 M $CaCl_2$ (Appendix II) and 0.1 ml of 10mg/ml thiamine hydrochloride (Appendix II). Finally, the soft overlay agar was transferred to a water bath to maintain the temperature of agar at 43°C to 45°C while six solid agar plates were pre-warmed in a 37°C incubator. Next, 4 ml of soft overlay agar was added to each of the 6 universals. The suspension was swirled to mix and then poured onto the pre-warmed solid agar plates to distribute the phage suspension in a thin even layer over the surface of the solid agar. The soft overlay was allowed to set at room temperature (15^{o}C to 25^{o}C). The plates were incubated aerobically at 37°C for 18-24 hours. Following incubation, 20 ml of #271 broth medium was added to each plate displaying clear confluent lysis as compared to the control plate. The soft agar surface was scraped off using a sterile spreader into 4 x 25 ml sterile glass universals, mixed vigorously and then centrifuged at 3,000 rpm for 25 minutes to sediment the cellular debris and agar. Finally, the supernatant containing the phage was passed through a 0.22 µm sterile 33 mm Millex-GP filter unit (Millipore, Watford, UK). The filtrate was stored at 2°C to 8°C or for prolonged storage at -20°C.

3.3.6.6. Determining plaque-forming units of *Escherichia coli* Phage MS2 by plating assays

In order to determine plaque-forming units (PFU), 200 µl of *E. coli* host was added to a sterile universal. This process was repeated 4 times. Four solid agar plates were pre-warmed in a 37°C incubator and 100 ml of soft overlay agar was melted in a microwave oven. The melted agar was cooled to approximately 50^0C before aseptically adding 1 ml of 10% glucose, 0.2 ml of 1 M $CaCl_2$ and 0.1 ml of 10mg/ml thiamine and then transferred to a water bath to maintain the temperature of the soft overlay agar at 43°C to 45°C. Next, 4 ml of soft agar was added to each universal. The contents were swirled to mix and then poured onto the pre-warmed solid agar plates to distribute the bacterial cells in a thin even layer over the surface of the solid agar. The soft agar overlay was allowed to set at room temperature (15°C to 25°C). A 10-fold serial dilution from 10^{-1} to 10^{-10} of MS2 bacteriophage was prepared in PBS (Appendix II). The solid agar plates with soft-agar overlay were divided into four quadrants. Using a fixed volume pipette, 25 µl of each dilution was transferred in duplicate onto the surface of the plates; two dilutions were put onto each plate. The plates were allowed to dry at room temperature (15°C to 25^oC). The plates were incubated aerobically at 37^0C for 18-24 hours. After incubation, the plates were examined for plaque formation. The number of plaques on each plate was counted and the titre of the original stock solution of phage (phage/ml) was calculated by multiplying the number of plaques by the dilution factor.

3.3.7. Optimisation of the human metapneumovirus two-step real-time RT-PCR assay

To perform the optimisation of the two-step real-time RT-PCR assay for HMPV detection using primer and probe sequences described by Maertzdorf *et al*. (2004) (Section 3.3.3.1) RNA template was first prepared from donated HMPV positive culture supernatant (Section 2.6.4.2) by manual purification using the QIAamp Viral RNA Mini Kit (QIAGEN Ltd, West Sussex, UK) (Section 2.6.4.3). In the next step, cDNA was generated using the TaqMan® Reverse Transcription Reagents (Applied Biosystems, Warrington, UK) in a final reaction volume of 20 µl with 1X RT Buffer, 5.5 mM $MgCl_2$, 500 µM each of dATP, dCTP, dGTP, dTTP, 2.5 µM random hexamers, 0.4 U/µl RNase inhibitor, and 1.25 U/µl Multiscribe Reverse Transcriptase (50 U/µl) (Section 2.6.6.1). The TaqMan® Universal PCR Master Mix without UNG AmpErase (Applied Biosystems, Warrington, UK) (Section 2.6.6.1) was used in conjunction with the cDNA template in a final reaction volume of 25 µl to determine the optimal primer and TaqMan® probe concentrations using an optimisation matrix (Section 2.6.6.4).

3.3.8. Sensitivity of the human metapneumovirus two-step real-time RT-PCR assay

The sensitivity of the two-step real-time RT-PCR for HMPV detection was established using donated positive virus culture supernatant that was serially diluted (10^{-1}, 10^{-2}, 10^{-3} to 10^{-10}) in a working solution of 1 ng/µl of cRNA (Section 2.6.6.3) before manual extraction using the QIAamp Viral RNA Mini Kit (QIAGEN Ltd, West Sussex, UK) (Section 2.6.4.3). Finally, the two-step real-time RT-PCR assay was performed (Section 2.6.6.1) in triplicate for each dilution using cDNA template generated from lower dilutions (10^{-3} to 10^{-10}) of positive virus culture supernatant.

3.3.9. A one-step real-time RT-PCR for streamlined human metapneumovirus detection

The two-step real-time RT-PCR for HMPV detection was improved further by streamlining the assay to a one-step protocol using the SuperScript™ III Platinum® One-Step qRT-PCR System (Invitrogen, Paisley, UK) (Section 2.6.6.2) in combination with forward and reverse primers and TaqMan® probe at concentrations determined from the optimisation of the two-step reaction. The one-step real-time RT-PCR assay was performed in a final reaction volume of 25 µl containing 12.5 µl of reaction buffer with a final magnesium sulphate ($MgSO_4$) concentration of 3 mM, 0.8µl Platinum® *Taq* DNA polymerase and 600 nM of NL-N forward primer, 200 nM of NL-N reverse primer, 200 nM of NL-N probe, and nuclease-free water (Bioline Ltd, London, UK) to a volume of 20 µl. Finally, 5 µl of RNA extract was added exclusive of the NTC. Instead, 5 µl of nuclease-free water (Bioline Ltd, London, UK) was added. The one-step RT-PCR real-time assay was performed on the Rotor-Gene™ 6000 series real-time analyser (Corbett Research Ltd, Cambridge, UK) using the thermal cycling parameters outlined in Section 2.6.6.2 and acquiring on the FAM channel.

The sensitivity of the one-step RT-PCR reaction for HMPV detection was established using donated positive virus culture supernatant that was serially diluted (10^{-1}, 10^{-2}, 10^{-3} to 10^{-10}) in a working solution of 1 ng/µl of cRNA (Section 2.6.6.3) before manual extraction using the QIAamp Viral RNA Mini Kit (QIAGEN Ltd, West Sussex, UK) (Section 2.6.4.3). The one-step real-time RT-PCR assay was performed (Section 2.6.6.2) in triplicate for each dilution using cDNA template generated from lower dilutions (10^{-3} to 10^{-10}) of positive virus culture supernatant.

3.3.10. Validation of novel primer and probe sequences to detect the Onderstepoort vaccine strain of canine distemper virus

Initial studies were conducted to determine the capacity of the novel primer and probe sequences to detect the Onderstepoort vaccine strain of canine distemper virus. RNA was extracted from virus previously grown on Vero cells (Section 3.3.6.4) using the QIAamp Viral RNA Mini Kit (QIAGEN Ltd, West Sussex, UK) (Section 2.6.4.3). Next, cDNA was generated using the TaqMan® Reverse Transcription Reagents (Applied Biosystems, Warrington, UK) in a final reaction volume of 20 µl with 1X RT Buffer, 5.5 mM $MgCl_2$, 500 µM each of dATP, dCTP, dGTP, dTTP, 2.5 µM random hexamers, 0.4 U/µl RNase inhibitor, and 1.25 U/µl Multiscribe Reverse Transcriptase (50 U/µl) (Section 2.6.6.1). Reverse transcription was performed on the GeneAmp 2400 Thermal Cycler (Perkin-Elmer, UK) (Section 2.6.6.1). The TaqMan® Universal PCR Master Mix without UNG AmpErase (Applied Biosystems, Warrington, UK) (Section 2.6.6.1) was used in combination with 400 nM of forward and reverse primers and 200 nM of TaqMan® probe. Oligonucleotide concentrations were not optimal but were suitable to allow assessment of the viability of the selected primer and probe sequences. Finally, 5 µl of cDNA template and nuclease-free water were added to a final reaction volume of 25 µl. Real-time PCR was performed on the ABI Prism® 7700 Real-Time Sequence Detection System (Applied Biosystems, Warrington, UK) (Section 2.6.6.1).

3.3.11. Optimisation of the Bacteriophage MS2 internal control assay

Optimisation of the MS2 bacteriophage detection system was performed using commercially available MS2 RNA (Roche Diagnostics Ltd, Burgess Hill, UK) that was supplied at a concentration of 0.8 µg/µl. A 10^{-6} dilution of MS2 RNA was prepared in a working solution of 1ng/µl of cRNA (Section 2.6.6.3) before manual purification using the QIAamp Viral RNA Mini Kit (QIAGEN Ltd, West Sussex, UK) (Section 2.6.4.3). Optimisation was performed using an optimisation matrix (Section 2.6.6.4) in conjunction with the SuperScript™ III Platinum® One-Step qRT-PCR System and RNA template in a final reaction volume of 25 µl on the Rotor-Gene™ 6000 series real-time analyser (Corbett Research Ltd, Cambridge, UK) using outlined thermal cycling parameters (Section 2.6.6.2).

3.3.12. Determination of optimal input concentration of MS2 bacteriophage per extraction

A 10-fold dilution series from 10^{-1} to 10^{-8} was prepared from the MS2 bacteriophage stock solution in PBS (Appendix II) in order to determine the optimal input concentration of MS2 bacteriophage required per nucleic acid extraction using the QIAamp Viral RNA Mini Kit

(QIAGEN Ltd, West Sussex, UK) (Section 2.6.4.3). Manual purification was performed in accordance with the manufacturer's instructions with the addition of 20 µl of internal control material at dilutions 10^{-1} to 10^{-8} to Buffer AVL alongside 140 µl of HMPV negative clinical material. Next, 5 µl of RNA template was added to a reaction mix containing the SuperScript™ III Platinum® One-Step qRT-PCR System in combination with primers and TaqMan® probe at optimal concentrations (Section 3.3.12). Real-time RT-PCR assay was performed on the Rotor-Gene™ 6000 series real-time analyser (Corbett Research Ltd, Cambridge, UK) using outlined thermal cycling parameters (Section 2.6.6.2).

3.3.13. Optimisation of Primer and Probe Concentrations for Duplex Reactions

The individual detection systems designed for HMPV and MS2 bacteriophage were combined in order to achieve simultaneous amplification of the two target sequences within a single reaction. To validate the change to a duplex format, a 10-fold dilution series was prepared from HMPV positive culture supernatant (Section 3.3.1) in a working solution of 1 ng/µl of cRNA (Section 2.6.6.3). The HMPV dilution series as well as a selection of HMPV positive respiratory specimens were prepared without MS2 bacteriophage and in combination with the optimal internal control input concentration by manual extraction using the QIAamp Viral RNA Mini Kit (QIAGEN Ltd, West Sussex, UK) (Section 2.6.4.3). Real-time RT-PCR was performed using the SuperScript™ III Platinum® One-Step qRT-PCR System in combination with HMPV primers and TaqMan® probe at optimised concentrations but the internal control oligonucleotide concentrations were reduced to ensure the duplex reaction favoured HMPV detection. Instead, primer and TaqMan® probe concentrations outlined in NSM VSOP 25 entitled "*Real-Time Quadriplex PCR for the Detection of Influenza*" (HPA, 2006) were used. The amended working reaction mix for duplex reactions is shown in Table 3.4.

Table 3.4. Preparation of working reaction mix for the human metapneumovirus real-time RT-PCR assay incorporating MS2 bacteriophage as the internal control.

Reagents	**Volume (µl) per 25 µl reaction**	**Stock Concentration**	**Final Concentration**
SuperScript III RT/Platinum® *Taq* Mix	12.5	-	-
2X Reaction Mix	0.80	2X	1X
NL-N forward primer	1.50	20 µM	600 nM
NL-N reverse primer	0.50	20 µM	200 nM
NL-N probe	1.00	10 µM	200 nM
MS2 forward primer	0.10	20 µM	80 nM
MS2 reverse primer	0.10	20 µM	80 nM
MS2 probe	0.20	10 µM	80 nM
Nuclease-free water	1.37	-	-
TOTAL VOLUME	20.00		

3.3.14. Evaluation of the COBAS AmpliPrep TNAI Kit for Automated Nucleic Acid Extraction

The performance of the COBAS® AmpliPrep TNAI Kit (Roche Diagnostics Ltd, Burgess Hill, UK) on the COBAS® AmpliPrep Instrument (Roche Diagnostics Ltd, Burgess Hill, UK) was validated for the preparation of highly purified nucleic acid from respiratory specimens including MS2 bacteriophage as an internal control in comparison to the QIAamp Viral RNA Mini Kit (QIAGEN Ltd, West Sussex, UK). In total, 128 archived, frozen respiratory specimens including 103 NPA, 16 throat swabs, 7 combined nose and throat swabs, 1 sputum and 1 BAL submitted to the Microbiology Department between September 2007 and April 2008 were included in the retrospective evaluation. All samples were examined routinely by virus culture, DFA (IMAGEN™, Oxoid Ltd, Hampshire, UK) for influenza virus types A and B, HRSV, PIV types 1-3, HRSV, adenovirus, and *Chlamydia* sp, and NOW® RSV Test (Binax, Inc., ME, USA) (Section 2.4.2). Total nucleic acid was extracted manually from 140 µl of sample material and eluted in a final volume of 60 µl using the QIAamp Viral RNA Mini Kit (QIAGEN Ltd, West Sussex, UK) (Section 2.6.4.3). A second 350 µl aliquot of all 128 specimens was extracted on the COBAS® AmpliPrep Instrument (Roche Diagnostics Ltd, Burgess Hill, UK) using the COBAS® AmpliPrep TNAI Kit (Roche Diagnostics Ltd, Burgess Hill, UK) with a final elution volume of 75 µl (Section 2.6.4.4). Twenty microlitres of MS2 bacteriophage at the optimal input concentration was included in the manual extraction procedure that was performed using the QIAamp Viral RNA Mini Kit (QIAGEN Ltd, West Sussex, UK) (Section 2.6.4.3). Additional internal control at the optimal input concentration was prepared from the MS2 bacteriophage stock solution in PBS (Appendix II). A minimum of 1.1 ml of working internal control was prepared for 12 tests and a maximum of 4.0 ml for 48 tests. Finally, the internal control was loaded onto the COBAS® AmpliPrep Instrument (Roche Diagnostics Ltd, Burgess Hill, UK). Qualitative real-time RT-PCR was performed for the viral targets HMPV, and all generic influenza virus A subtypes (H1-H15) and influenza virus B (Section 3.3.4) using the SuperScript™ III Platinum® One-Step qRT-PCR System on the Rotor-Gene™ 6000 series real-time analyser (Corbett Research Ltd, Cambridge, UK) (Section 2.6.6.2).

3.3.15. Agarose Gel Electrophoresis

Amplification products of expected size were visualised by gel electrophoresis on a 2% (w/v) agarose gel. The agarose gel was prepared by gently heating 1 g of UltraPure™ Agarose (Invitrogen, Paisley, UK) in 50 ml of 1X Tris-Borate-EDTA (TBE) buffer (Ambion®, Warrington, UK) (Appendix II) until dissolved. The molten agarose gel was cooled to 60°C before adding 1 µl of 10 mg/ml ethidium bromide solution (Sigma-Aldrich, Dorset, UK). An 8-well comb was

inserted into gel casting plate about 5 to 10 mm from the end of the plate. The agarose gel was gently poured into a 7 x 10 cm UV-transparent gel tray (Bio-Rad, Hertfordshire, UK). Bubbles were removed. The gel was allowed to set for approximately 30 minutes at room temperature (15^{o}C to 25^{o}C). Once the gel was set, the comb was removed and the gel tray carefully placed into the Mini-Sub Cell GT Cell Tank (Bio-Rad, Hertfordshire, UK). The gel tank was carefully filled with 1X TBE buffer (Appendix II) ensuring that no air remained in the wells by washing with 1X TBE.

Samples were prepared by adding 1 µl of ready-to-use 5X DNA Loading Buffer (Bioline Ltd, London, UK) to 4 µl of PCR product. Next, 5 µl of each prepared DNA sample was loaded alongside 5 µl of HyperLadder™ V, a ready-to use 25 bp molecular weight marker (Bioline Ltd, London, UK). Each marker contained multiple standard bands of between 40 ng to 80 ng of DNA and two intensive bands of 120 ng of DNA to facilitate DNA quantification and size DNA fragments. The gel was run at a constant voltage of 120 V/cm until the dye had migrated approximately half to three quarters of the length of the gel. DNA fragments were visualised under ultra-violet (UV) light using the UVP BioDoc-it™ Imaging System (Ultra-Violet Products Ltd, Cambridge, UK) and the gel image captured using Polaroid film.

3.3.16. Cloning real-time RT-PCR products

Amplification products purified using the QIAquick® PCR purification kit (QIAGEN Ltd, Crawley, West Sussex, UK) were cloned into the plasmid vector pCR®2.1-TOPO® using the TOPO TA Cloning® Kit (Invitrogen, Paisley, UK) and transformed into One Shot® Mach1™-T1® Chemically Competent *Escherichia coli* (Invitrogen, Paisley, UK) subsequent to confirmation of the presence of a single discrete band by agarose gel electrophoresis.

3.3.16.1. Addition of 3’ A-Overhangs Post-Amplification

The SuperScript™ III One-Step RT-PCR System utilised in the one-step real-time RT-PCR assay for HMPV detection uses Platinum® Taq DNA Polymerase High Fidelity, an enzyme mixture composed of recombinant Taq DNA polymerase, *Pyrococcus* species GB-D polymerase, and Platinum® Taq antibodies. *Pyrococcus* species GB-D polymerase is a proofreading enzyme that possesses a 3’ to 5’ exonuclease activity. Direct cloning of DNA amplified by proofreading polymerases into TOPO TA Cloning® vectors is often difficult because proofreading polymerases remove the unpaired 3’ adenine nucleotides necessary for TA Cloning®. *Taq* polymerase was used to ensure the presence of 3’adenine (A) overhangs on

the PCR product to permit TA Cloning of blunt-ended fragments. The addition of 3' A-overhangs was performed in a final reaction volume of 100 µl containing 90 µl of fresh PCR product, 0.5 µl *Taq* DNA polymerase, and 0.5 µl of dNTPs. The reaction was incubated for 10 minutes at 72°C on a PTC-200 DNA Engine Peltier Thermal Cycler (MJ Research, Inc., Massachusetts, USA). At the end of incubation the samples were placed on ice before treatment the QIAquick® PCR purification kit (QIAGEN, Crawley, West Sussex, UK).

3.3.16.2. Setting up the Cloning Reaction

With the desired PCR product ready, the next step was to clone the product into the pCR®2.1-TOPO® using the TOPO TA Cloning® Kit (Invitrogen, Paisley, UK). The TOPO® Cloning reaction for eventual transformation into chemically competent TOP10F' One Shot® *E. coli* was performed according to manufacturer's instructions. Briefly, 1 µl, 2 µl or 4 µl of fresh PCR product, 1 µl of salt solution, and 1 µl of TOPO® vector were combined in a 0.2 ml reaction tube (Alpha Laboratories, Hampshire, UK) with nuclease-free water to a final reaction volume of 6 µl. The tubes were mixed gently before incubation at room temperature (15°C to 25°C) for 5 minutes. At the end of incubation the samples were placed on ice until required to transform the pCR®2.1-TOPO® construct into competent *E. coli*.

3.3.16.3. Transforming One Shot® Mach1™-T1^R Competent Cells

Transformations using the Mach1™-T1^R strain allowed visualisation of colonies 8 hours after plating on ampicillin selective plates or overnight after plating on kanamycin selective plates. A vial of One Shot® Mach1™-T1^R Chemically Competent *E. coli* was thawed on ice for each transformation. The pUC19 control plasmid DNA supplied with the kit was used to verify the efficiency of the transformation experiment. Next, 2 µl of the prepared plasmid DNA was added to a vial of One Shot® cells and 1 µl of pUC19 control was added to a separate vial of One Shot® cells. The vials were mixed gently, incubated on ice for 30 minutes and then placed in a water-bath equilibrated to 42°C for 40 seconds in order to heat-shock the cells. The vials were removed from the water bath and immediately placed on ice for 2 minutes. Next, 250 µl of room temperature 2x YT Microbial Medium (Appendix II) was added to each vial. Each vial was closed tightly before the cells were incubated at 37°C for 1 hour with horizontal shaking at 225 rpm. In the interim, LB agar plates containing 50 µg/ml of ampicillin or kanamycin (Appendix II) were warmed at 30^{o}C for 30 minutes. Next, the LB agar plates were spread with 40 µl of 40 µg/ml 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL) solution (Melford Laboratories Ltd, Suffolk, UK) (Appendix II). The

plates were incubated at 37°C until required. After incubation, 2 different volumes of transformation mix, 25 µl, and 50 µl, was spread onto separate pre-warmed LB agar plates to ensure that well-spaced colonies grew on at least one plate. After overnight incubation at 37°C, the plates were stored at 4°C to allow the blue colour to develop fully.

3.3.16.4. Cultivation of transformants

Individual white or pale blue colonies were selected for culture in 5 ml of pre-warmed LB medium containing 50 µg/ml of ampicillin or kanamycin (Appendix II). The LB medium was incubated in an orbital shaker at 37°C for 16 hours with vertical shaking at 220 rpm.

3.3.17. Purification of Plasmid DNA

On removal from the orbital shaker, the cultures were centrifuged at 5000 rpm for 10 minutes to pellet the bacterial cells. The supernatant was removed and the pelleted bacterial cells were resuspended in 250 µl of Buffer P1, containing a final concentration of 100 µg/ml of RNase A solution and LyseBlue reagent, a simple visual identification system to prevent common handling errors that lead to inefficient cell lysis and incomplete precipitation of SDS, cell debris, and genomic DNA. LyseBlue formed a precipitate after addition to Buffer P1 at a ratio of 1:1000. This was the first stage in the purification of plasmid DNA using the QIAprep® Miniprep Kit (QIAGEN, Crawley, West Sussex, UK), which was performed in accordance with the manufacturer's instructions. All centrifugation steps were performed at 13,000 rpm in a conventional, tabletop microcentrifuge. The cell suspension was transferred to a 1.5 ml microcentrifuge tube (Alpha Laboratories, Hampshire, UK) and following the addition of 250 µl of lysis buffer, Buffer P2, turned blue as the LyseBlue precipitate dissolved. The cell suspension was mixed thoroughly by inverting the tube six times to produce a homogenously coloured suspension. Next, 350 µl of Buffer N3 was added. The cell suspension mixed thoroughly immediately after the addition of the N3 neutralisation buffer by inverting the tube 6 times until all trace of blue disappeared and a homogenous colourless suspension remained that indicated that the SDS from the lysis buffer had been precipitated effectively. The suspension was centrifuged for 10 minutes to form a compact white pellet. A QIAprep spin column was placed in a 1.2 ml collection tube and the supernatant was applied to QIAprep spin column by pipetting. The column was centrifuged for 60 seconds and the through-flow was discarded. The QIAprep spin column was placed back into the same collection tube and 0.5 ml of Buffer PB was added to the QIAprep spin column. The spin column was centrifuged for 60 seconds and the through-flow

was discarded. This brief wash step was necessary to remove trace endonuclease activity that was associated with using *endA+* and wildtype strains with high nuclease activity such as the Mach1™-T1^{R} *E. coli* strain to prevent degradation of the plasmid DNA. The QIAprep spin column was placed back into the same collection tube and 0.75 ml of ethanol-containing Buffer PE was added. The column was centrifuged for 60 seconds in a second brief wash step to remove salts. The flow-through was discarded and the QIAprep column was placed back in the same collection tube. The column was centrifuged for an additional 60 seconds to remove any residual Buffer PE that may inhibit subsequent enzymatic reactions. After centrifugation, the QIAprep spin column was opened for 60 seconds to allow any remaining ethanol to evaporate. Finally, the column was placed into a clean 1.5 ml microcentrifuge tube (Alpha Laboratories, Hampshire, UK) and 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) was dispensed directly onto the centre of the QIAprep membrane for optimal elution of bound plasmid DNA. The column was incubated at room temperature (15°C to 25°C) for 60 seconds before a final centrifugation step for 60 seconds. The purified DNA was stored at –20°C until required.

3.3.18. Restriction enzyme digestion analysis of plasmid DNA

A typical restriction digest reaction containing 1 µg of plasmid DNA, 2.5 µl of 5X SuRE/Cut Buffer H (Roche Diagnostics Ltd, East Sussex, UK), 1 unit of *Eco*R I (Roche Diagnostics Ltd, East Sussex, UK) and nuclease-free water (Bioline, London, UK) to a final reaction volume of 25 µl were combined in a 0.2 ml reaction tube (Alpha Laboratories, Hampshire, UK). The contents were mixed by pipetting up and down and centrifuged briefly at 12,000 x*g* to collect the contents at the bottom of the tube. The reaction was incubated for 1 hour at 37°C on a PTC-200 DNA Engine Peltier Thermal Cycler (MJ Research, Inc., Massachusetts, USA) before *Eco*R I was heat-inactivated by incubating at 65°C for 15 minutes. At the end of incubation, 6 µl of 5X DNA loading buffer (Bioline, London, UK) was added to the resultant products of the restriction enzyme digestion reaction and analysed by agarose gel electrophoresis on a 2% agarose gel containing 10 mg/ml ethidium bromide solution as described previously.

3.3.19. Sequence analysis of constructs

The construct was sequenced by Genome Enterprise Limited, The Genome Analysis Centre, Norwich Research Park, Norwich, UK, using the M13 Forward primer (-20) (5´-GTAAAACGACGGCCAG-3') included in the TOPO TA Cloning® Kit (Invitrogen, Paisley, UK) in

combination with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) on the 3730*xl* DNA Analyser (Applied Biosystems, Warrington, UK).

3.3.20. Long-term storage of plasmids

A glycerol stock of the transformants was prepared for long-term storage once the correct clone was identified. The original colony was streaked out on LB plates containing 50 µg/ml kanamycin. A single colony was inoculated into 2 ml of LB medium containing 50 µg/ml kanamycin. Bacterial cells were grown until the culture reached stationary phase. Next, 0.85 ml of bacterial culture was combined with 0.15 ml of sterile glycerol (Invitrogen, Paisley, UK) and transferred to a cryovial (Alpha Laboratories, Hampshire, UK). The culture was stored at -80°C for prolonged storage.

3.4. Results

3.4.1. A streamlined real-time RT-PCR assay for human metapneumovirus detection

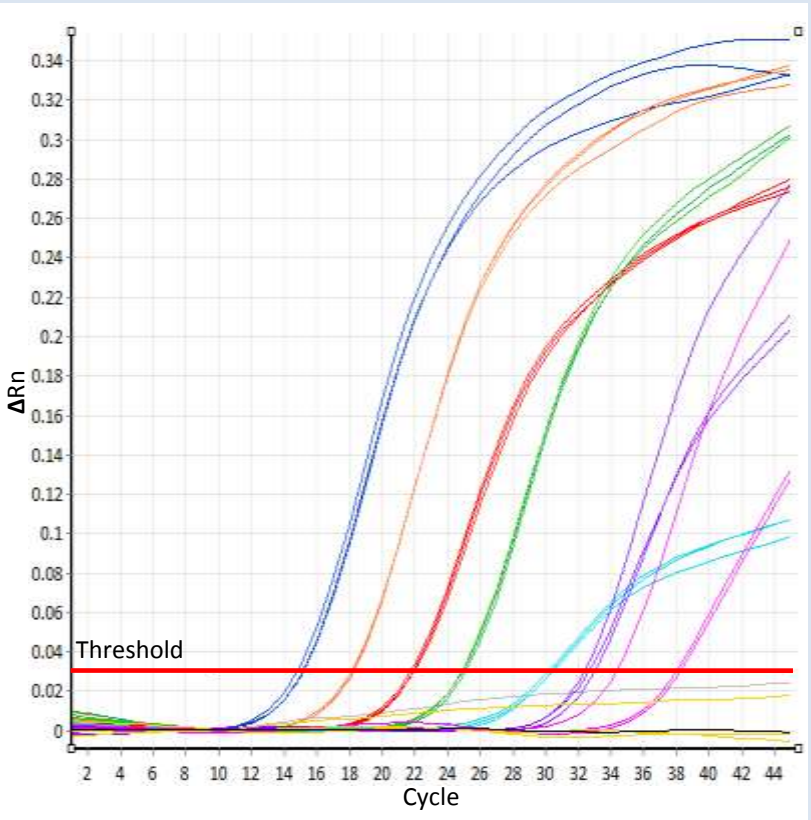
The fully optimised conditions for the two-step real-time RT-PCR assay for HMPV detection on the ABI Prism® 7700 Real-Time Sequence Detection System (Applied Biosystems, Warrington, UK) are outlined in Table 3.5.

Table 3.5. Optimised conditions for a two-step real-time RT-PCR assay for the detection of human metapneumovirus on the ABI Prism® 7700 Real-Time Sequence Detection System.

Reagents	**Volume (µl) per sample**	**Stock Concentration**	**Final Concentration**
TaqMan® Universal PCR Master Mix	12.5	2X	1X
NL-N forward primer	0.75	20 µM	600 nM
NL-N reverse primer	0.25	20 µM	200 nM
NL-N probe	0.50	10 µM	200 nM
Nuclease-free water	6.00	-	-
TOTAL VOLUME	**20.00**		

The optimisation was performed to determine the minimum primer and probe concentrations to ensure the generation of specific, repeatable, reproducible, and sensitive data. The sensitivity of the two-step real-time RT-PCR for HMPV detection was thoroughly examined by Maertzdorf *et al*., (2004) using serially diluted RNA from virus stocks and RNA runoff transcripts from the N protein gene of four prototype HMPV strains. Hence, only minimum experiments were performed here to ensure that the two-step real-time RT-PCR assay worked well within a different laboratory setting. The lowest reproducibly detected

dilution was determined as the cut-off (Figure 3.5). The two-step real-time RT-PCR assay demonstrated consistent detection capabilities over the range of dilutions investigated (10^{-3} to 10^{-10}) (Figure 3.5). The final detection limit of the two-step real-time RT-PCR assay was attained at the 10^{-9} dilution.



	Dilution	C_T
	Water	Negative
	10^{-3}	15.14
	10^{-3}	14.80
	10^{-3}	15.16
	10^{-4}	18.17
	10^{-4}	18.25
	10^{-4}	18.11
	10^{-5}	21.76
	10^{-5}	21.91
	10^{-5}	21.96
	10^{-6}	24.92
	10^{-6}	24.86
	10^{-6}	25.11
	10^{-7}	30.17
	10^{-7}	30.44
	10^{-7}	30.37
	10^{-8}	32.35
	10^{-8}	32.63
	10^{-8}	33.00
	10^{-9}	34.34
	10^{-9}	38.01
	10^{-9}	38.20
	10^{-10}	Negative
	10^{-10}	Negative
	10^{-10}	Negative
	NTC	Negative

Figure 3.5. The sensitivity of the two-step real-time RT-PCR for human metapneumovirus detection was performed using 10-fold serial dilutions of positive virus culture supernatant from 10^{-3} to 10^{-10}. All dilutions were detected successfully excepting the 10-^{10} dilution.

The two-step real-time RT-PCR assay for HMPV detection was improved further by streamlining the assay to a one-step protocol using the SuperScript™ III Platinum® One-Step qRT-PCR System (Invitrogen, Paisley, UK) in combination with forward and reverse primers and TaqMan® probe at optimised concentrations determined for the two-step real-time RT-PCR assay. Comparison of C_T values between the two-step and one-step real-time RT-PCR assays was performed to ensure that the change to a one-step protocol did not have a negative influence on the sensitivity of the assay. The C_T values of the HMPV dilution series in the one-step protocol were within +/- 1 cycle of those of the two-step real-time RT-PCR assay (Table 3.6).

Table 3.6. Comparison of cycle threshold (C_T) values between the two-step and one-step real-time RT-PCR assays for the detection of human metapneumovirus on the ABI Prism® 7700 Real-Time Sequence Detection System and Rotor-Gene™ 6000 series real-time analyser, respectively.

Virus dilution	Mean C_T values		Difference in C_T values (+/-)
	Two-step real-time RT-PCR	One-step real-time RT-PCR	
10^{-3}	15.03	15.69	+0.66
10^{-4}	18.18	19.06	+0.88
10^{-5}	21.88	22.74	+0.86
10^{-6}	24.96	25.21	+0.25
10^{-7}	30.33	29.85	-0.48
10^{-8}	32.66	33.27	+0.61
10^{-9}	36.85	36.12	-0.73
10^{-10}	Negative	Negative	-

3.4.2. Internal control strategies

3.4.2.1. Optimisation of the Bacteriophage MS2 internal control assay

The fully optimised conditions for MS2 bacteriophage detection on the Rotor-Gene™ 6000 series real-time analyser (Corbett Research Ltd, Cambridge, UK) achieved using commercially available MS2 RNA (Roche Diagnostics Ltd, Burgess Hill, UK) are outlined in Table 3.7.

Table 3.7. Optimised conditions for a one-step real-time RT-PCR assay for the detection of MS2 bacteriophage on the Rotor-Gene™ 6000 series real-time analyser.

Reagents	Volume (µl) per sample	Stock Concentration	Final Concentration
SuperScript III RT/Platinum® *Taq* Mix	12.5	-	-
2X Reaction Mix	0.8	2X	1X
MS2 forward primer	0.1	20 µM	50 nM
MS2 reverse primer	0.3	20 µM	200 nM
MS2 probe	0.3	10 µM	100 nM
Nuclease-free water	6.1	-	-
TOTAL VOLUME	20.00		

3.4.2.2. Optimal input concentration of MS2 bacteriophage per extraction

The titre of the original stock solution of MS2 bacteriophage (phage/ml) was calculated by multiplying the number of plaques by the dilution factor following plating assays. Plaques were too numerous to count at dilutions 10^{-1} to 10^{-7} and were discounted. In contrast, plaques were no longer visible at the 10^{-10} dilution. However, plaques were clearly visible at dilutions 10^{-8} and 10^{-9} and so the stock solution of MS2 phage was calculated as 24 x 10^7 pfu/ml and 3 x 10^8 pfu/ml, respectively. The optimal input concentration of MS2

bacteriophage required per nucleic extraction was ascertained through manual extraction of MS2 bacteriophage at dilutions 10^{-1} to 10^{-8} alongside HMPV negative clinical material. All MS2 bacteriophage dilutions were detected successfully but only the 10^{-6} and 10^{-7} dilutions offered suitability for inclusion in nucleic acid extraction alongside the primary target. MS2 bacteriophage at dilutions 10^{-1} to 10^{-5} was detected at C_T values <25 indicating that the internal control target was too abundant and may interfere with amplification of the primary target. In contrast, MS2 bacteriophage was detected at a C_T value >30 at the lowest dilution, which reduced the reliability of the internal control (Figure 3.6).

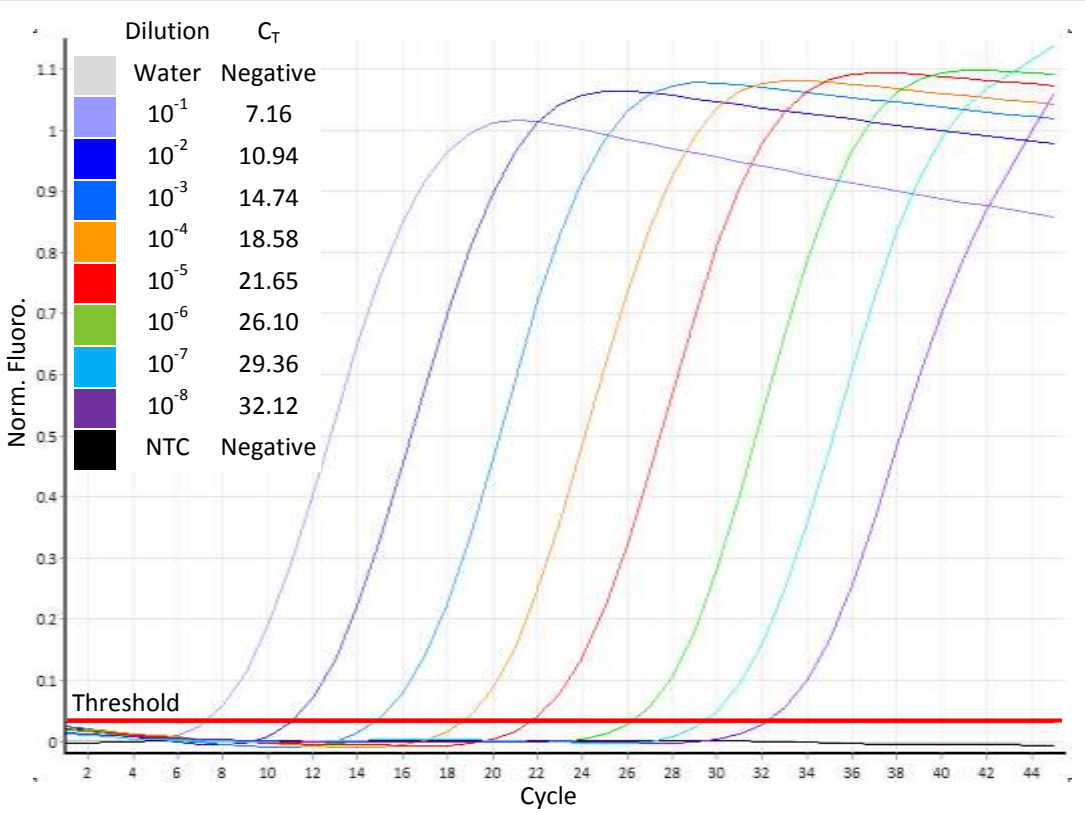
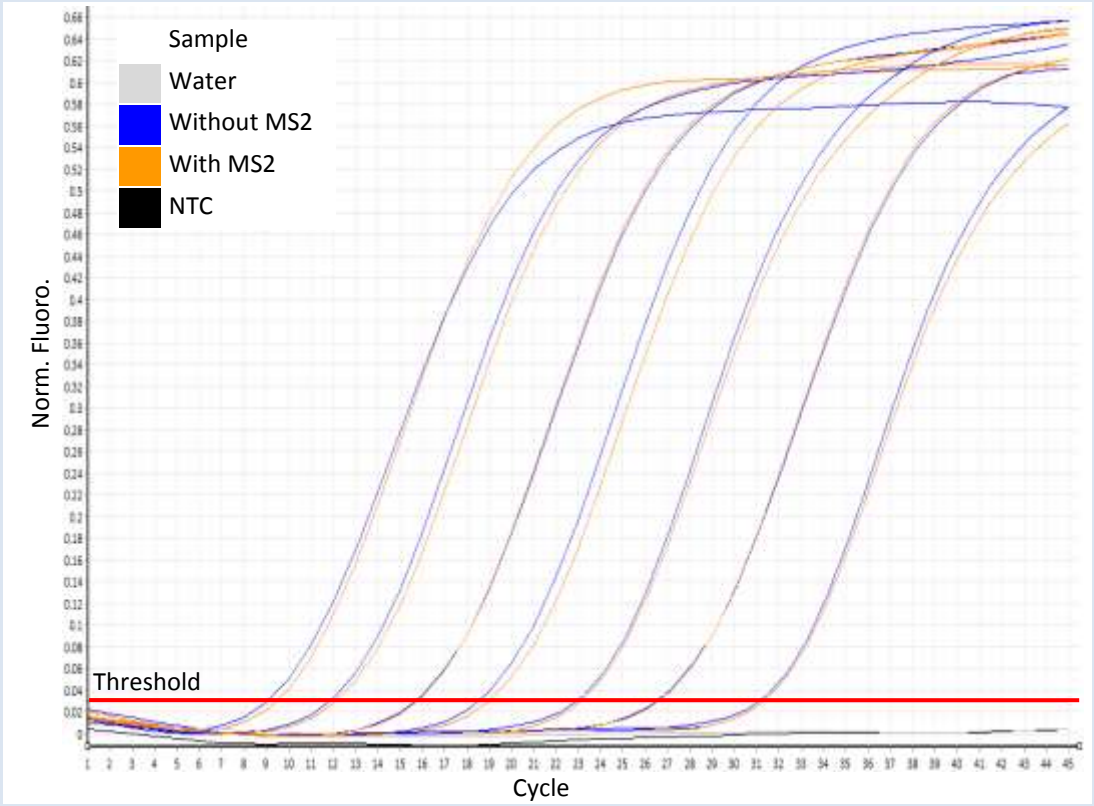
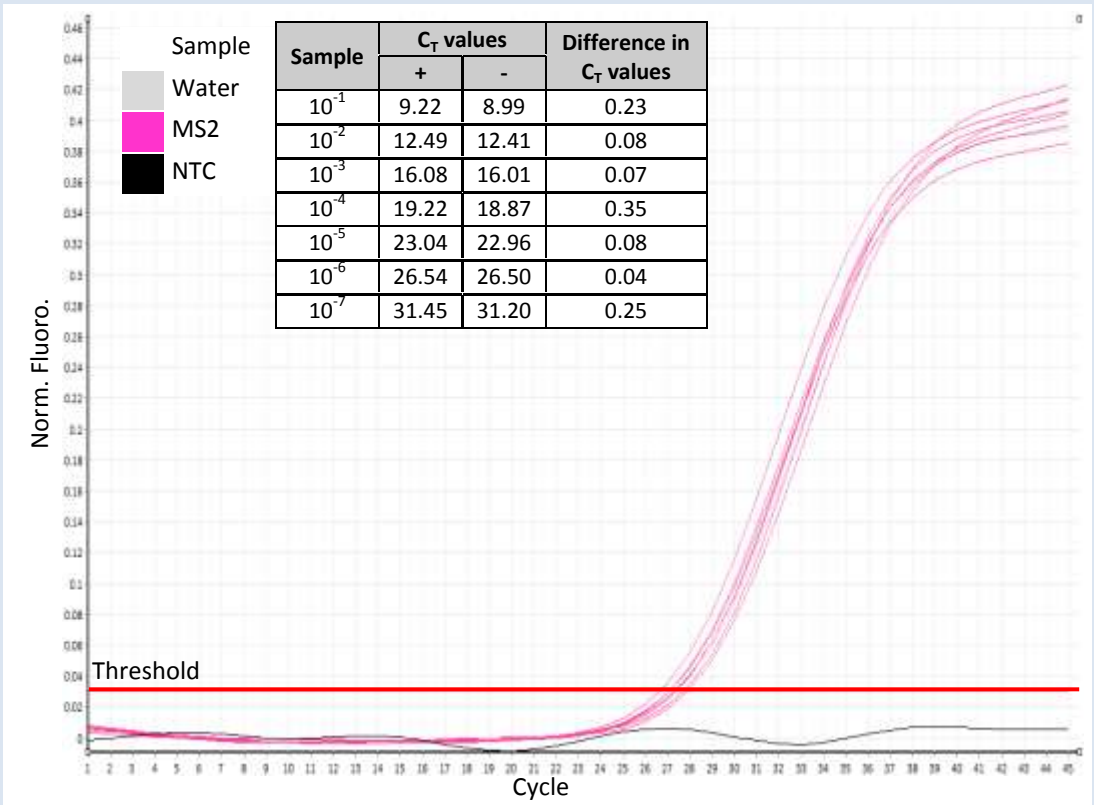


Figure 3.6. Detemination of the optimal input concentration of MS2 bacteriophage required per nucleic extraction was ascertained using eight 10-fold serial dilutions prepared from the MS2 bacteriophage stock solution in PBS, which was co-extracted with human metapneumovirus negative clinical material using the QIAamp Viral RNA Mini Kit.

3.4.2.3. Combination of the detection systems designed for HMPV and MS2 bacteriophage

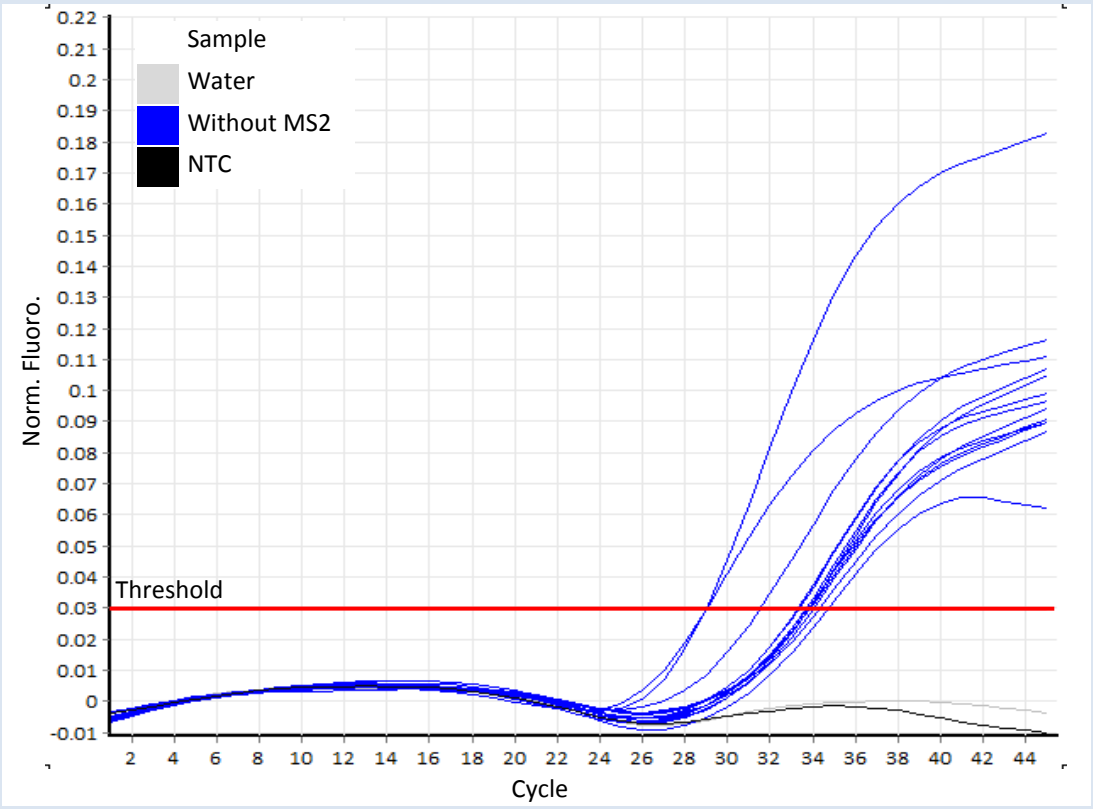
No detrimental effect was observed on the sensitivity of the primary target within the duplex assay in comparison to the monoplex assay subsequent to the inclusion of MS2 bacteriophage as the internal control target since only minor variation in C_T values was observed between dilutions (Figure 3.8) and respiratory specimens (Figure 3.9) that were amplified with and without MS2 bacteriophage.

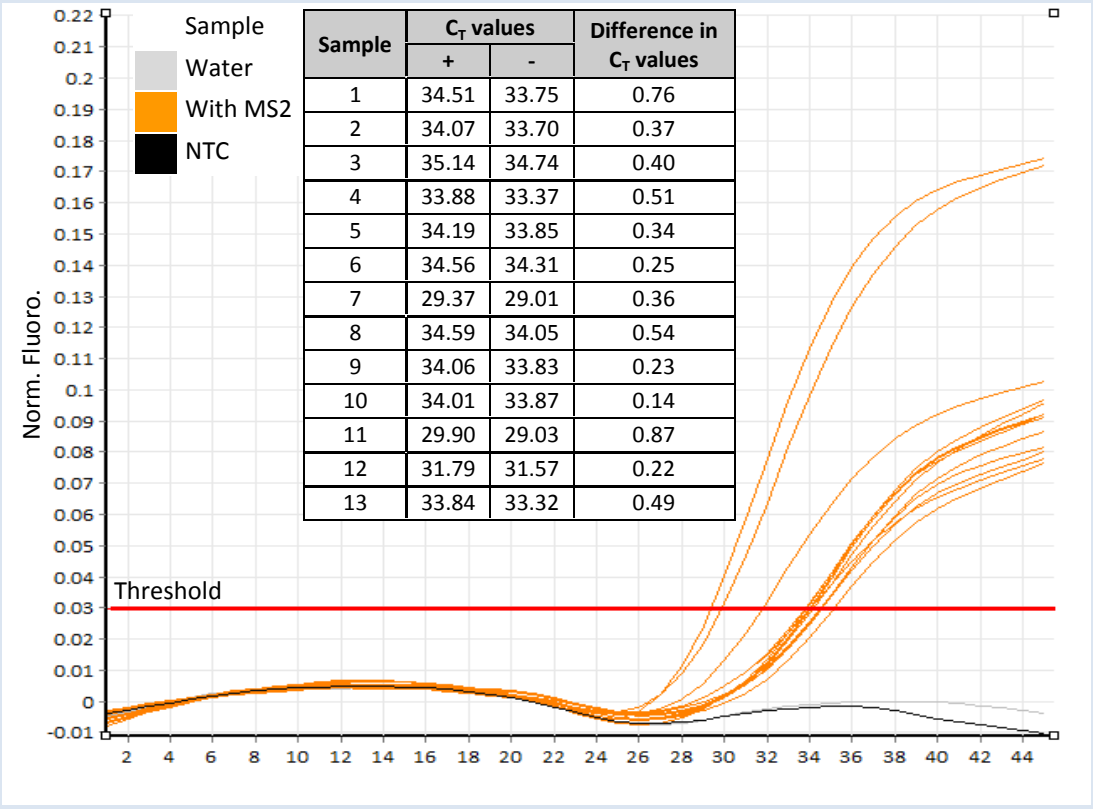




Sample	C_T values		Difference in C_T values
	+	-	
10^{-1}	9.22	8.99	0.23
10^{-2}	12.49	12.41	0.08
10^{-3}	16.08	16.01	0.07
10^{-4}	19.22	18.87	0.35
10^{-5}	23.04	22.96	0.08
10^{-6}	26.54	26.50	0.04
10^{-7}	31.45	31.20	0.25

Figure 3.7. Comparison of cycle threshold (C_T) values of serially diluted human metapneumovirus positive culture supernatant with and without MS2 bacteriophage to determine the effect of the addition of the internal control target on detection of human metapneumovirus in the duplex real-time RT-PCR assay. Only minimum differences in C_T values were observed between dilutions amplified with and without MS2 bacteriophage.





Sample	C_T values		Difference in C_T values
	+	-	
1	34.51	33.75	0.76
2	34.07	33.70	0.37
3	35.14	34.74	0.40
4	33.88	33.37	0.51
5	34.19	33.85	0.34
6	34.56	34.31	0.25
7	29.37	29.01	0.36
8	34.59	34.05	0.54
9	34.06	33.83	0.23
10	34.01	33.87	0.14
11	29.90	29.03	0.87
12	31.79	31.57	0.22
13	33.84	33.32	0.49

Figure 3.8. Comparison of cycle threshold (C_T) values among human metapneumovirus positive clinical samples with and without MS2 bacteriophage to determine the effect of the addition of the internal control target on detection of human metapneumovirus in the duplex real-time RT-PCR assay. The difference in C_T values for each clinical specimen with and without MS2 bacteriophage is shown in the table.

3.4.2.4. Detection of the Onderstepoort vaccine strain of canine distemper virus

Initial studies demonstrated that the novel primer and probe sequences selected within the N gene of the Onderstepoort vaccine strain of canine distemper virus successfully detected the virus (Figure 3.7). However, no further work was performed on this target subsequent to these preliminary investigations. The MS2 bacteriophage was selected as the preferential target for inclusion in the real-time RT-PCR assay for HMPV detection.

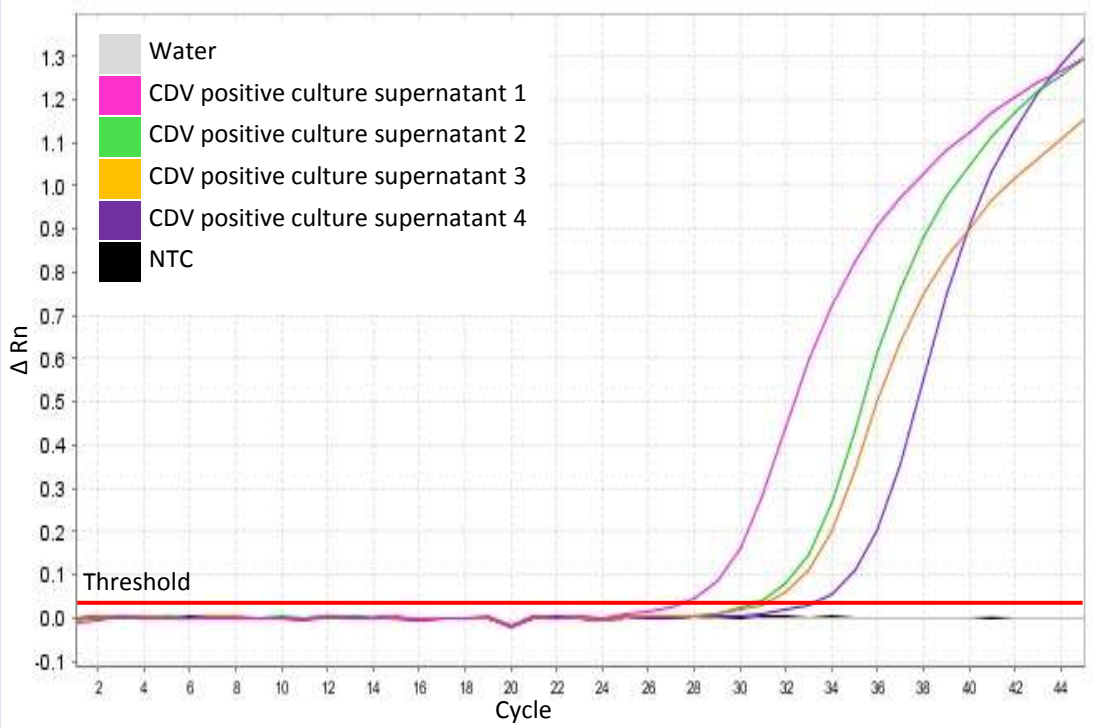


Figure 3.9. Amplification plots showing the cycle threshold (C_T) values obtained for the Onderstepoort vaccine strain of canine distemper virus (CDV) demonstrating the ability of the novel primer and probe sequences targeting the nucleoprotein gene to detect the virus. Validation studies were performed using virus grown on confluent continuous cell culture monolayers of Vero cells.

3.4.3. Evaluation of the COBAS AmpliPrep TNAI Kit for Automated Nucleic Acid Extraction

The evaluation of the performance of the COBAS® AmpliPrep TNAI Kit (Roche Diagnostics Ltd, Burgess Hill, UK) for automated nucleic acid extraction on the COBAS® AmpliPrep Instrument (Roche Diagnostics Ltd, Burgess Hill, UK) included 128 archived, frozen respiratory specimens. Qualitative real-time RT-PCR was performed for the viral targets HMPV, influenza virus type A, and influenza virus type B. HMPV was detected in 9 (7.0%) of respiratory specimens by real-time RT-PCR assay while influenza virus type A and influenza virus type B were detected by the real-time quadriplex PCR in 7 (5.5%) and 9 (7.0%) respiratory specimens, respectively. The C_T values obtained for positive respiratory

specimens following extraction by the COBAS® AmpliPrep TNAI Kit (Roche Diagnostics Ltd, Burgess Hill, UK) on the COBAS® AmpliPrep Instrument (Roche Diagnostics Ltd, Burgess Hill, UK) in comparison to the QIAamp Viral RNA Mini Kit (QIAGEN Ltd, West Sussex, UK) varied between -1.33 and 2.40 with an average variance of 0.68 (Table 3.8).

Table 3.8. Comparison of crossing threshold (C_T) values obtained for positive respiratory specimens following automated extraction by the COBAS® AmpliPrep TNAI Kit on the COBAS® AmpliPrep Instrument and manual extraction by the QIAamp Viral RNA Mini Kit.

VIrus	Sample	COBAS® AmpliPrep TNAI Kit		QIAamp Viral RNA Mini Kit		Difference in C_T values (+/-)
		Status	C_T	Status	C_T	
HMPV	49/07	Positive	32.75	Positive	31.75	1
	51/07	Positive	26.93	Positive	28.08	-1.15
	52/07	Positive	26.89	Positive	25.89	1
	12/08	Positive	31.65	Positive	30.59	1.06
	13/08	Positive	26.83	Positive	24.58	2.25
	15/08	Positive	35.95	Positive	35.16	0.79
	45/08	Positive	26.97	Positive	25.76	1.21
	55/08	Positive	20.28	Positive	21.61	-1.33
	59/08	Positive	34.52	Positive	32.12	2.40
FA	56/07	Positive	23.68	Positive	22.95	0.73
	58/07	Positive	21.85	Positive	20.87	0.98
	01/08	Positive	37.25	Positive	37.93	-0.68
	14/08	Positive	23.34	Positive	22.67	0.67
	17/08	Positive	26.29	Positive	24.56	1.73
	30/08	Positive	35.82	Positive	36.37	-0.55
	58/08	Positive	26.52	Positive	27.69	-1.17
FB	24/08	Positive	30.53	Positive	29.66	0.87
	28/08	Positive	35.50	Positive	34.27	1.23
	31/08	Positive	26.30	Positive	23.97	2.33
	44/08	Positive	20.54	Positive	19.67	0.87
	46/08	Positive	30.54	Positive	29.43	1.11
	49/08	Positive	22.38	Positive	23.14	-0.76
	51/08	Positive	24.95	Positive	24.14	0.81
	65/08	Positive	14.78	Positive	14.14	0.64
	66/08	Positive	27.73	Positive	26.70	1.03

3.4.4. Plasmid generation as an alternative human metapneumovirus positive control

A plasmid offered an alternative source of control material in the absence of a commercial working reagent for HMPV. The plasmid was generated by cloning the 170 bp region within the HMPV N gene into the vector pCR®2.1-TOPO®. Sequencing was performed using the M13 Forward primer included in the TOPO TA Cloning® Kit (Invitrogen, Paisley, UK) (Section 3.3.19) to confirm that the gene was cloned in the correct orientation. Restriction

endonuclease digestion of plasmid DNA was performed to confirm the presence and correct orientation of the insert using *Eco*R I, a class II restriction enzyme (Section 3.3.18). A glycerol stock of the transformants was prepared for long-term storage at -80°C once the correct clone was identified (Section 3.3.20).

3.5. Discussion

The exploration of molecular diagnostic techniques for the detection of viruses has heralded major changes within the routine diagnostic virology setting, which is classically defined by the use of virus culture to grow viruses (Carman and Niesters, 2007). This has particular relevance to the Microbiology Department at the Norfolk and Norwich University Hospital, which is embracing a move from traditional virus culture to molecular diagnostic techniques for diagnosis of respiratory virus infection. The introduction of a real-time RT-PCR assay for HMPV detection marked the inception of the molecular diagnostic repertoire. This molecular diagnostic test was based on primer and probe sequences described by Maertzdorf *et al*. (2004), which demonstrated the capacity to detect HMPV from the four genetic lineages with equal sensitivity and specificity in comparison to previously described assays (Mackay *et al*., 2003; van den Hoogen *et al*., 2003). Though novel primer and probe sequences were not designed for the purpose of this study, improvements to the original assay described by Maertzdorf *et al*. (2004) were undertaken. The substitution of the quencher molecule, TAMRA (6-carboxy-tetramethylrhodamine) with BHQ-1 at the 3' end of the TaqMan® probe was the only modification made to the original primer and probe sequences described by Maertzdorf *et al*. (2004). Nevertheless, this substitution was performed due to the inherent fluorescence properties of TAMRA that result in a relatively poor signal-to-noise ratio. Moreover, the original two-step real-time RT-PCR assay described by Maertzdorf *et al*. (2004) was streamlined into a routine diagnostic friendly one-step RT-PCR assay using the SuperScript™ III Platinum® One-Step qRT-PCR System (Invitrogen Ltd, Paisley, UK). Finally, MS2 bacteriophage was incorporated successfully as an internal control. Although at one time it was considered that the use of a one-step method offered reduced sensitivity in contrast to a two-step method, the findings within the present study do not support this generalisation, which perhaps reflects improvements in the enzymes used in one-step kits. In fact, the one-step real-time RT-PCR protocol offered many advantages over the two-step format including reduced hands-on time as well as opportunities for error and contamination. The successful implementation of the streamlined one-step RT-PCR assay for HMPV detection also increased the feasibility of implementing other in-house designed

molecular diagnostic systems for the diagnosis of respiratory virus infections within the routine diagnostic setting, which is restricted by turnaround times, since multiple targets can be detected in a single tube using the same thermocycling parameters.

The use of a second target nucleic acid, which serves as an internal control has become an important strategy in diagnostic PCR to combat the inherent problems associated with the presence of amplification inhibitors in clinical specimens (Dreier *et al*., 2005). Obtaining a positive signal from the second target demonstrates successful nucleic acid extraction and amplification, thereby validating a negative result for the primary target (Rosenstraus *et al*., 1998). Many different types of internal control have been utilised (Hoorfar *et al*., 2004; Espy *et al*., 2006). Ideally, an internal control for diagnostic RT-PCR assays should be straightforward and economical to produce and standardise and should have sufficient stability for routine storage and use. Its sequence should be absent from clinical samples, and it should be suitable for many different assays, the results of which should be simple to interpret (Dingle *et al*., 2004). It was considered that the Onderstepoort strain of canine distemper virus would fulfil all such criteria as demonstrated previously by the inclusion of animal viruses as universal internal DNA and RNA controls (Cleland *et al*., 1999; Mattison *et al*., 2009; Comelli *et al*., 2008; Clancy *et al.*, 2008; van Doornum *et al*., 2003). Moreover, the virus was selected for investigation as a novel internal control for inclusion in the diagnostic RT-PCR assay based on the assumption that as a RNA virus and a member of the family *Paramyxoviridae* it would behave more similarly in the extraction procedure to the target virus. However, the production of animal viruses raises issues of safety (Dingle *et al*., 2004; Drier *et al*., 2005). Furthermore, the foreseeable end to the use of virus culture within the routine diagnostic setting compelled consideration of other favourable options. The *E. coli* Phage MS2 presented an ideal alternative to the Onderstepoort strain of canine distemper virus as it is prone to the same inhibition/degradation as RNA viruses, is non-infectious, stable, and easily propagated (Rolfe *et al*., 2007). The versatility of MS2 bacteriophage as an internal control is increasingly described (Blaise-Boisseau *et al*., 2010; Dreier *et al*, 2005; Ellis and Curran, 2010; Ninove *et al*., 2011; Rolfe *et al*., 2007), which is perhaps also reflected in the inclusion of MS2 bacteriophage as an internal control in the xTAG® respiratory virus panel (RVP) test (Luminex Molecular Diagnostics Inc., Toronto, Canada), a commercial diagnostic test for the detection of multiple respiratory viruses and subtypes. It was decided that bacteriophage MS2 would offer greater suitability as an internal control than the Onderstepoort strain of canine distemper virus after consideration of issues of safety related to the propagation of an animal virus and the uncertain future of virus culture facilities

within the Microbiology Department. MS2 bacteriophage was incorporated successfully as an internal control into all subsequent in-house designed molecular diagnostic systems in order to enhance the quality of routine molecular diagnosis provided by the Microbiology Department (Ninove *et al*., 2011). MS2 bacteriophage has offered great viability, which has ensured its continued utilisation as an internal control within the Microbiology Department. It is relatively easy to produce new stocks of MS2 bacteriophage although a commercial MS2 RNA is available. Moreover, MS2 bacteriophage offers excellent stability for long-term storage. Indeed, stock has been stored at -70°C for 4 years with no increase in the concentration required in the nucleic acid extraction.

It is without a doubt that the successful implementation of molecular diagnostic systems for the detection of HMPV and other respiratory viruses into routine molecular diagnostic service within a demanding laboratory would have been more challenging without an automated system for nucleic acid extraction. The COBAS® AmpliPrep Instrument in combination with the COBAS® TaqMan 48 Analyser (Roche Diagnostics Ltd, Burgess Hill, UK) was purchased originally for quantitation of HIV-1 and Hepatitis C virus (HCV) RNA. However, the availability of the COBAS® AmpliPrep TNAI Kit for the preparation of highly purified total nucleic acid on the COBAS® AmpliPrep Instrument made automation of nucleic acid extraction a feasible option. The COBAS® AmpliPrep TNAI Kit presented a major advantage over the QIAamp Viral RNA Mini Kit (QIAGEN Ltd, West Sussex, UK) as PCR reactions for DNA and RNA viruses could be performed from the same nucleic acid eluate. Moreover, the COBAS® AmpliPrep TNAI Kit on the COBAS® AmpliPrep Instrument reduced the overall processing time for 24 samples from 180 minutes using the manual QIAamp Viral RNA Mini Kit to 120 minutes, offered freedom from this otherwise laborious and time-consuming task, enabled standardisation of the process and limited human error and contamination. Utilisation of the COBAS® AmpliPrep Instrument in combination with the COBAS® AmpliPrep TNAI Kit for the extraction of total nucleic acid from a range of respiratory specimen types presented a novel application, which subsequently gained interest from other clinical laboratories as well as a request to present the results of a preliminary evaluation at a sponsored symposium. Although recently superseded by newer more sensitive nucleic acid extraction platforms, the COBAS® AmpliPrep Instrument in combination with the COBAS® AmpliPrep TNAI Kit provided comparable sensitivity to the manual QIAamp Viral RNA Mini Kit and enabled successful automation of nucleic acid extraction when the molecular diagnostic service was in its infancy.

One of the most difficult aspects with undertaking a research project on a new virus is obtaining positive control material. The virus could not be easily grown using cell lines available within the routine diagnostic setting and no commercial positive control material was available early in the discovery of the virus. Colleagues at the Specialist Virology Centre, Gartnavel General Hospital, Glasgow, Scotland made the early stages in the development of the real-time RT-PCR assay for HMPV detection possible until positive clinical specimens were identified. The generation of a plasmid seemed a positive alternative at the beginning of this study and although the plasmid was generated successfully, it was after all a creation of PCR product, which does not provide a useful source of control material in molecular techniques that are designed for the detection of RNA viruses. Furthermore, no suitable facilities for the generation of new plasmid were available within the Microbiology Department to prevent the contamination of other areas dedicated to molecular diagnostics. The plasmid is not in routine use within the routine diagnostic setting. At present, all positive control material is derived from residual positive respiratory specimens.

In conclusion, the real-time RT-PCR assay for HMPV detection was improved successfully by streamlining the assay into a one-step format, which offered greater flexibility while the inclusion of MS2 bacteriophage as an internal control addressed inherent problems associated with the presence of PCR inhibitors and so raised confidence in results generated for the viral target of interest. The successful implementation of the real-time RT-PCR assay for HMPV detection within a demanding clinical virology laboratory was aided by the replacement of the manual nucleic acid extraction procedure with an automated method, which offered additional benefits of reduced processing and hand-on time, enabled standardisation of the process and limited human error and contamination. The success of this molecular diagnostic system has been demonstrated through participation in the external quality assessment programme, Quality Control for Molecular Diagnostics (QCMD) since 2008, which has seen a 100% pass rate and subsequent development of the service to include other respiratory viruses.

CHAPTER FOUR

4. Frequent involvement of human metapneumovirus with *Haemophilus influenzae* in respiratory infection in children

4.1. Introduction

Many respiratory viruses share seasonality and susceptible populations (Sloots *et al*., 2008). So, it is not surprising that co-infection of HMPV with other respiratory viruses is widely reported (Al-Sonboli *et al*., 2005; Bosis *et al*., 2005; Canducci *et al*., 2008; Caracciolo *et al*., 2008; Choi *et al*., 2006; Cilla *et al*., 2008; Cuevas *et al*., 2003; De Paulis *et al*., 2011; Foulonge *et al*., 2006; Greensill *et al*. 2003; Pilger *et al*., 2011; Sánchez-Yebra *et al*., 2011; Sasaki *et al*., 2005; Sung *et al*., 2011; Viazov *et al*. 2003; Wolf *et al*., 2006; Xepapadaki *et al*., 2004; Xiao *et al*., 2010). However, the importance of co-infection with HMPV remains inconclusive at present. Several studies have presented evidence to implicate co-infection with HMPV as a marker of severity (Greensill *et al*., 2003, König *et al*., 2004; Semple *et al*., 2005). Greensill *et al*. (2003) reported 70% (21/30) of infants requiring admission to the Paediatric Intensive Care Unit (PICU) with severe HRSV bronchiolitis were co-infected with HMPV. Subsequent demonstration that dual infection with HMPV and HRSV conferred a 10-fold increase in the relative risk (RR) of admission to PICU for mechanical ventilation confirmed an association between severe disease and co-infection by HMPV and HRSV (Semple *et al*., 2005). However, support for a cumulative pathogenic effect on disease severity conferred by this viral partnership is limited (Ali *et al*., 2010; Foulongne *et al*., 2006; König *et al*., 2004) and conflicted by evidence that suggests that HMPV co-infection is uncommon in severe HRSV-associated RTI (De Paulis *et al*., 2011; Lazar *et al*., 2004; Sung *et al*., 2011; van Woensel *et al*., 2006). So the chance of a co-infection appears more stochastical than representative of a specific relationship between two pathogens at present (Weigl *et al*., 2007). Likewise evidence of co-infection of HMPV with other respiratory viruses is not associated with increased disease severity (Falchi *et al*., 2011; García-García *et al*., 2006a; Sloots *et al*., 2006; Wilkesmann *et al*., 2006; Wolf *et al*., 2006). However, statistical evidence strongly supports an interaction between HMPV and HRSV subtype A and B in ARTI (Brunstein *et al*., 2008).

While the debate on the interaction between HMPV and other respiratory viruses continues, few studies have directly considered the importance of bacterial co-infection in HMPV-associated RTI (Ampofo *et al*. 2008; Kukavica-Ibrulj *et al*., 2009; Lehtinen *et al*., 2006; Lin *et al*., 2005; Ludewick *et al*., 2011; Madhi *et al*., 2006; Verkaik *et al*., 2011) despite the well documented importance of respiratory viruses in facilitating secondary bacterial infections (Ampofo *et al*., 2008; Kim *et al*., 1996; Talbot *et al*., 2005). The earliest published scientific

work to examine the importance of bacterial co-infection in the pathogenesis of HMPV was conducted in South Africa in a cohort of children randomised to receive a 9-valent pneumococcal conjugate vaccine (PCV9) or placebo (Madhi *et al*., 2006). NPA samples were tested for the presence of HMPV when children within the cohort were hospitalised with LRTI (Madhi *et al*., 2006). Among fully vaccinated HIV–uninfected children, the incidence of HMPV-associated LRTI was reduced by 45%, and the incidence of clinical pneumonia was reduced by 55%. Similarly, in fully vaccinated HIV-infected children, the incidence of HMPV-associated LRTI was reduced by 53%, and that of clinical pneumonia was reduced by 65% suggesting that co-infection with *S. pneumoniae* is an essential part of the pathogenesis of most severe HMPV infections progressing to pneumonia (Madhi *et al*., 2006).

Interactions of different infectious agents often modify the outcome of an infection, compared to the course of infections caused by only a single agent (Degré, 1986). Viral infection is one common factor that can upset the delicate balance of the respiratory tract and the combined viral-bacterial infection aggravates the clinical disease to predispose to subsequent secondary bacterial superinfection (Degré, 1986). To investigate the potential synergistic interaction of HMPV with *S. pneumoniae*, an experimental murine model was established that closely mimicked the clinical exacerbation of HMPV-associated respiratory disease by *S. pneumoniae* (Kukavica-Ibrulj *et al*., 2009). Four-to-six week old BALB/c mice were infected intranasally on day 0 with low viral inocula of HMPV that would not produce overt clinical symptoms followed by a superinfection 5 days later with a non-lethal dose of *S. pneumoniae*. Pulmonary cytokine and chemokine levels were measured in the lungs of sacrificed mice on days 6, 7, and 8 after virus infection, corresponding to 24, 48, and 72 hours following bacterial superinfection, respectively (Kukavica-Ibrulj *et al*., 2009). Evident from this study was the concept that infection with HMPV creates an environment in the respiratory tract that predisposes the host to an exacerbated response to subsequent bacterial infections, akin to influenza A virus (Kukavica-Ibrulj *et al*., 2009). Mice with bacterial superinfection demonstrated significant weight loss and higher levels of airway obstruction compared to mice infected with HMPV, or pneumococcus alone. Bacterial counts increased from 5 x 10^2 colony-forming units (CFU)/lung in mice infected with pneumococcus only to 10^7 and 10^9 CFU/lung in mice with prior infections with HMPV (Kukavica-Ibrulj *et al*., 2009). The low viral inocula used for initial infection resulted in a minimal increase of the proinflammatory cytokines and chemokines expressed in lungs of infected mice. In contrast, the lungs of superinfected mice displayed a dramatic increase in pulmonary levels of the proinflammatory cytokines tumor necrosis factor-alpha (TNF-α),

interferon-gamma (IFN-γ), interleukin-1 α (IL-1α), interleukin-1 beta (IL-1β), IL-6 and IL-12 (p70) as well as the anti-inflammatory cytokine IL-13 and the chemokines macrophage inflammatory protein 1α (MIP-1α), monocyte chemotactic protein 1 (MCP-1), the mouse IL-8 homologue KC. Of particular note was the very important increase of granulocyte colony-stimulating factor (G-CSF) and, to a lesser extent, granulocyte-monocyte colony-stimulating factor (GM-CSF) in lungs of superinfected mice (Kukavica-Ibrulj *et al*., 2009). G-CSF, which regulates the maturation, differentiation, and proliferation of neutrophils, may increase neutrophil survival, allowing the sustained secretion of tissue-damaging molecules in the lungs of superinfected animals, which together with high pulmonary levels of proinflammatory cytokines likely contribute to the enhanced pneumococcal disease seen after HMPV virus infection (Kukavica-Ibrulj *et al*., 2009). Hence, these results suggest that multiple inflammatory mediators are synergistically exacerbated and contribute to the morbidity and mortality seen in the murine model (Kukavica-Ibrulj *et al*., 2009). However, this synergistic interaction is limited to the period of active HMPV replication in contrast to the long-term effects caused by influenza A virus (Ludewick *et al*., 2011).

While virus infection in the respiratory tract is usually perceived to pave the way for subsequent bacterial superinfection (Degré, 1986) it is unusual to consider that bacterial exposure can influence susceptibility to virus infection. However, recent studies suggest that exposure to *S. pneumoniae* is associated with increased susceptibility to HMPV infection (Verkaik *et al*., 2011). Serum samples and colonisation data for *H. influenzae*, *M. catarrhalis*, *S. aureus* and *S. pneumoniae* were collected from 57 children at 1.5, 6, 14 and 24 months of age (Verkaik *et al*., 2011). Seroconversion rates to HMPV were determined and related to bacterial carriage. Frequent nasopharyngeal carriage (≥2 times in the first two years of life) of *S. pneumoniae*, but not of the other three pathogens, was associated with increased seroconversion rates of infants to HMPV at the age of two (frequently vs. less exposed, 93% vs. 59%; $P<0.05$) (Verkaik *et al*., 2011). Subsequently, the susceptibility of well-differentiated normal human bronchial epithelial cells (wd-NHBE) pre-incubated with bacterial pathogens to *in vitro* HMPV infection was evaluated (Verkaik *et al*., 2011). Pre-incubation of wd-NHBE with *S. pneumoniae* resulted in increased susceptibility to infection with HMPV-enhanced green fluorescent protein (EGFP), as determined by enumeration of EGFP-positive cells (Verkaik *et al*., 2011). This was not the case for cells pre-incubated with *H. influenzae*, *M. catarrhalis*, and *S. aureus* (Verkaik *et al*., 2011). The combined *in vivo* and *in vitro* data suggest that exposure to *S. pneumoniae* can modulate HMPV infection (Verkaik *et al*., 2011). Nevertheless, the specific interaction between *S. pneumoniae* and HMPV infection that

contributes to the modification in the clinical outcome of HMPV infection is purely speculative at present. Pneumococcal virulence and immune evasive factors, including capsule or pneumolysin, may facilitate HMPV infection in multiple ways (Verkaik *et al*., 2011). First, these factors may penetrate the mucus layer and inhibit ciliary beating of respiratory epithelial cells. This could expose susceptible human epithelial cells, resulting in enhancement of HMPV infection or spread. In addition, influx or activation of immune cells residing in or associated with the respiratory epithelium may facilitate HMPV infection (Verkaik *et al*., 2011). Moreover, bacterial factors stimulating pulmonary toll-like receptor 2 (TLR2) and TLR4 responses may provoke an enhanced immune response following HMPV infection (Verkaik *et al*., 2011). Furthermore, lipopeptides in the bacterial cell wall may lead to enhanced viral binding to target cells, facilitating HMPV infection, and spread (Verkaik *et al*., 2011). In addition, pneumococcal immune evasive factors counteract host innate immune responses, which may also facilitate HMPV infection (Verkaik *et al*., 2011).

4.2. Aims

Current studies support the role of HMPV in the development of invasive pneumococcal pneumonia. However, evidence is lacking to form a complete picture of the relationship between HMPV and other bacteria that are habitually implicated in mixed viral-bacterial respiratory tract infections. These include *H. influenzae*, *M. catarrhalis*, *S. aureus*, and *Streptococcus pyogenes*. The aim of this study was to determine the frequency of bacterial and viral co-infections in children hospitalised with symptoms of ARTI in order to provide evidence to support a potential role for other commensal flora of the nasopharynx in co-infections with HMPV.

4.3. Materials and Methods

4.3.1. Overview of study design

This retrospective, cross-sectional study included 726 frozen, archived NPA samples preserved in VTM that were collected between 31st October 2005 and 31st December 2008 from children $\leq$18 years attending the Norfolk and Norwich University Hospital with symptoms of ARTI and submitted to the Microbiology Department for investigations for microbial causes of respiratory tract infection. All samples were examined routinely by virus culture, DFA (IMAGEN^{TM}, Oxoid Ltd, Hampshire, UK), and NOW$^{®}$ RSV Test (Binax, Inc., ME, USA) for influenza virus types A and B, HRSV, PIV types 1-3, HRSV, adenovirus, and

Chlamydia sp and cultured on appropriate bacteriological media for the isolation of target organisms including *H. influenzae*, *M. catarrhalis*, *S. aureus*, *S. pneumoniae* (Section 2.4).

All samples were assigned a laboratory accession number that was unique to each routine diagnostic investigation and linked to patient record data via the iSOFT TelePath computer system. Demographic information relating to age, gender, date of sample collection, routine investigations performed as well as test results was sought in the computer system in order to generate a database within Microsoft Excel and SPSS version 17.0 (SPSS Inc, Chicago, Illinois, USA) before anonymisation of archived NPA samples in preparation for subsequent comprehensive retrospective screening for HMPV using molecular methodology.

4.3.2. Ethical Approval

Permission to undertake retrospective screening of residual NPA samples for HMPV was granted by the East Norfolk and Waveney Research Governance Committee and the Norfolk Research Ethics Committee (REC Reference number: 05/Q0101/77) (Appendix I).

4.3.3. Retrospective screening for human metapneumovirus

All samples were subjected to nucleic acid extraction using the COBAS® AmpliPrep TNAI Kit (Roche Diagnostics Ltd, Burgess Hill, UK) on the COBAS® AmpliPrep Instrument (Roche Diagnostics Ltd, Burgess Hill, UK) (Section 3.3). Total nucleic acid was screened for the presence of HMPV by duplex one-step real-time RT-PCR on the Rotor-Gene™ 6000 series real-time analyser (Corbett Research UK Ltd, Cambridge, UK) (Section 3.3) using established primer and probes sequences designed according to Maertzdorf *et al*. (2004) (Section 3.3).

4.3.4. Statistical analysis

Characteristics and outcomes according to pathogen were compared using Spearman's rank correlation coefficient and the Mann-Whitney *U* test. Statistical calculations were performed with SPSS version 17.0 (SPSS Inc, Chicago, Illinois, USA). A p value of <0.05 was considered statistically significant.

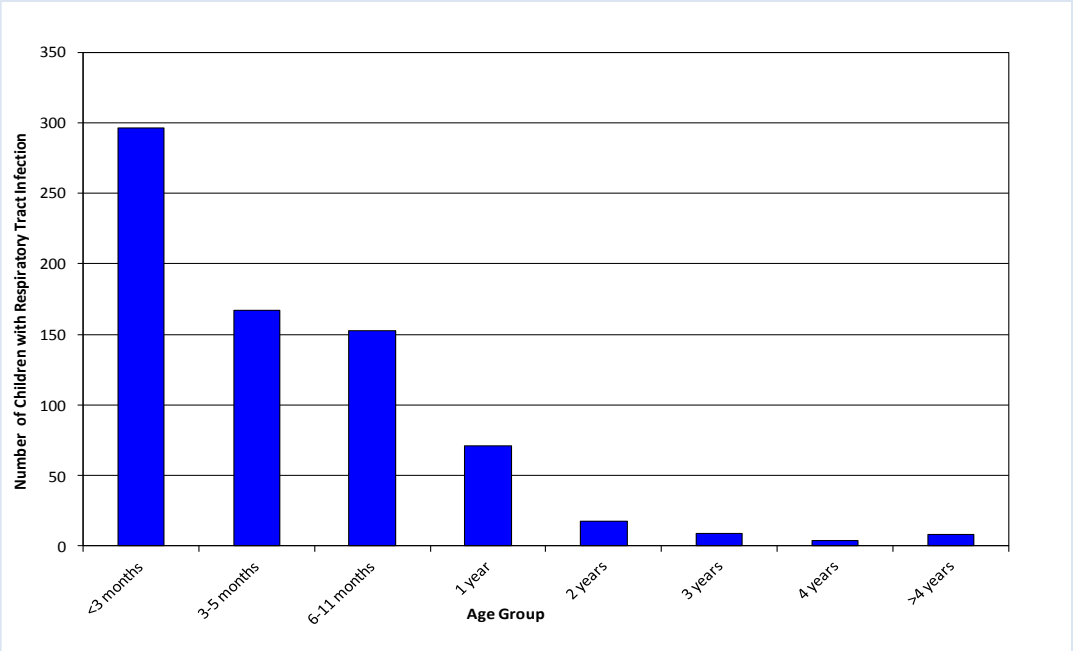
4.4. Results

4.4.1. Characteristics of patients and specimens tested

The sample series consisted of 821 frozen, archived NPA samples collected between 31st October 2005 and 31st December 2008 from children ≤18 years attending the Norfolk and Norwich University Hospital between 31st October 2005 and 31st December 2008 with symptoms of ARTI. Of these, 726 (88.4%) samples were included in this retrospective study; comprehensive virological and bacteriological screening was incomplete for the remainder of the archived samples.

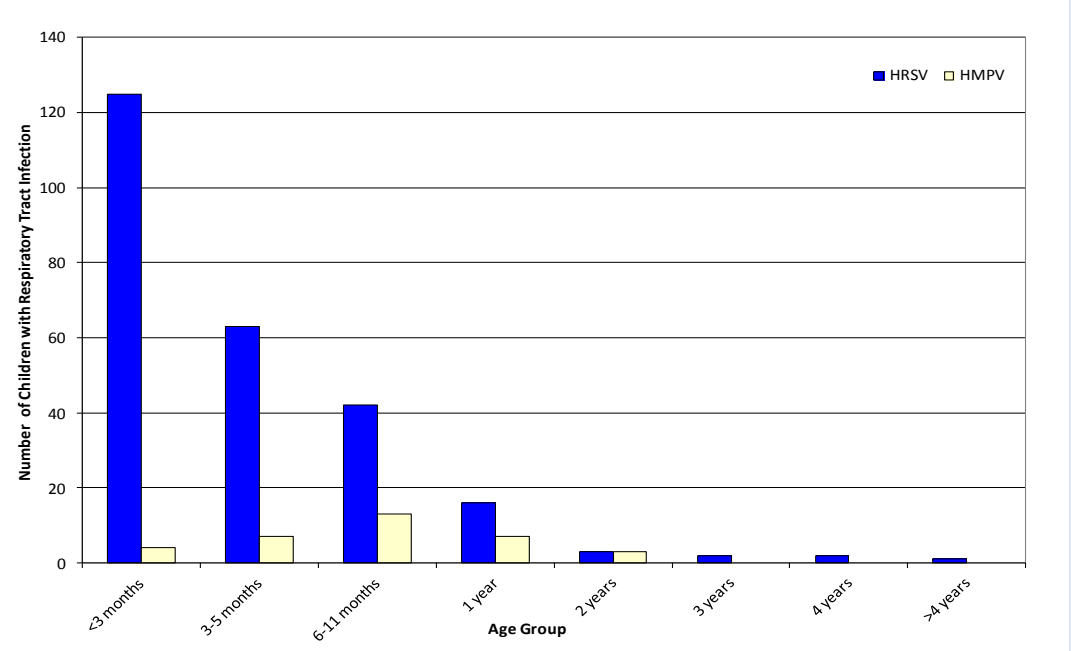
Rates of hospitalisation for RTI were highest in males (442/726; 60.9%) compared to females (284/726; 39.1%). A higher percentage of RTI was observed among males with HRSV (157/726; 21.6%) or HMPV (24/726; 3.3%) infection than females with HRSV (100/726; 13.8%) or HMPV (14/726; 1.9%) infection. Ages ranged from 0 to 144 months with a mean and median age of 6.8 months and 4.1 months, respectively. The majority of episodes of ARTI occurred in paediatric patients aged <1 year (616/726; 84.8%). The incidence of RTI was most acute among paediatric patients <3 months (Figure 4.1).

Figure 4.1. Age distribution of children hospitalised for acute respiratory tract infection.



More than 90% of all respiratory tract infections attributable to HMPV and HRSV occurred in the first year of life (Figure 5.2). Children with HMPV-associated RTI were older in comparison to children with HRSV respiratory infection (Figure 5.2). The peak age for HRSV infections was less than 3 months and 6 to 11 months for HMPV infections (Figure 4.2).

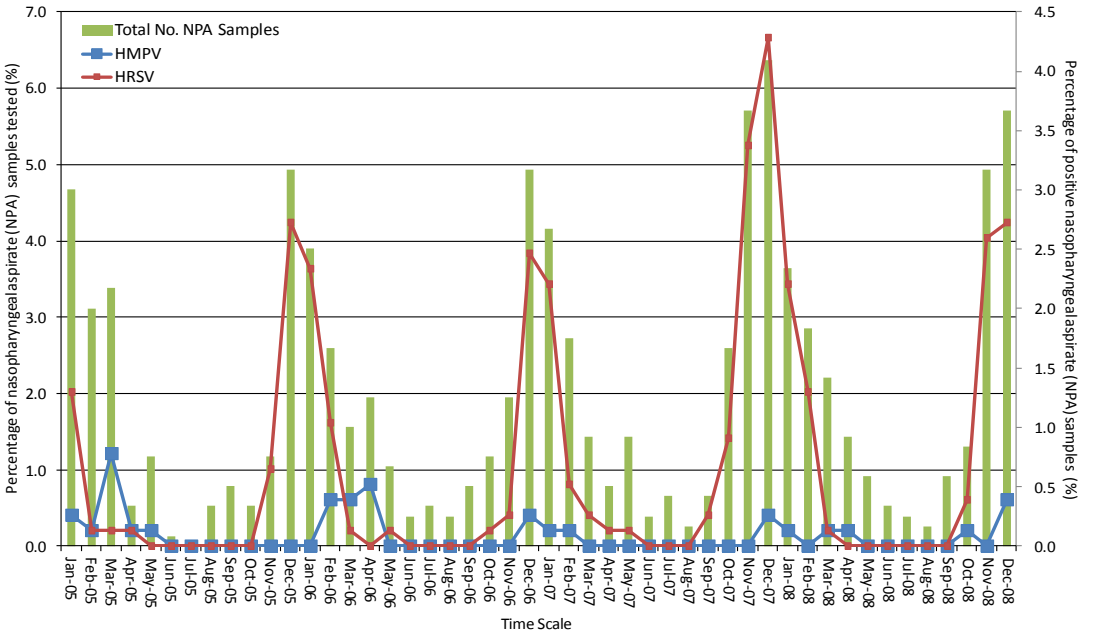
Figure 4.2. Age distribution of paediatric patients infected with human respiratory syncytial virus and human metapneumovirus.



Abbreviations: HRSV, human respiratory syncytial virus; HMPV, human metapneumovirus

The majority of NPA samples included in this retrospective, cross-sectional study were submitted during the winter season (October–March) (Figure 4.3). It is clear that HMPV co-circulates with HRSV. A partial overlap with the annual HRSV epidemic occurred during 2005/2006 and 2007/2008 but coincided completely with seasonal HRSV activity during 2006/2007 with peak HMPV activity mirroring peak HRSV activity during seasons 2006/2007 and 2007/2008 (Figure 4.3). Nevertheless, it is also of worth noting that the seasonal circulation of HMPV between 31st October 2005 and 31st December 2008 among paediatric patients with acute respiratory symptoms also differed from the circulation pattern of HRSV. Circulation of HRSV commenced in early autumn within this paediatric cohort with annual peaks of infection occurring in winter (Figure 4.3). In contrast, identification of the first HMPV infections occurred several months after the start of the HRSV epidemic with the majority of HMPV infections occurring in winter and spring (Figure 4.3). HMPV infections were limited in autumn and no HMPV infections occurred during the summer months of all three consecutive years. While there is no clear biennial pattern of alternate winter versus spring HMPV activity, the incidence of the virus within this paediatric cohort is markedly more conspicuous in spring 2006 and 2008 in contrast to spring 2007 with infection occurring within this paediatric cohort until April (Figure 4.3).

Figure 4.3. Comparison of the seasonal circulation patterns of human respiratory syncytial virus and human metapneumovirus among nasopharyngeal aspirate samples submitted to the Microbiology Department, Norfolk & Norwich University Hospital between 31st October 2005 and 31st December.



Abbreviations: HRSV, human respiratory syncytial virus; HMPV, human metapneumovirus

4.4.2. Respiratory Pathogens

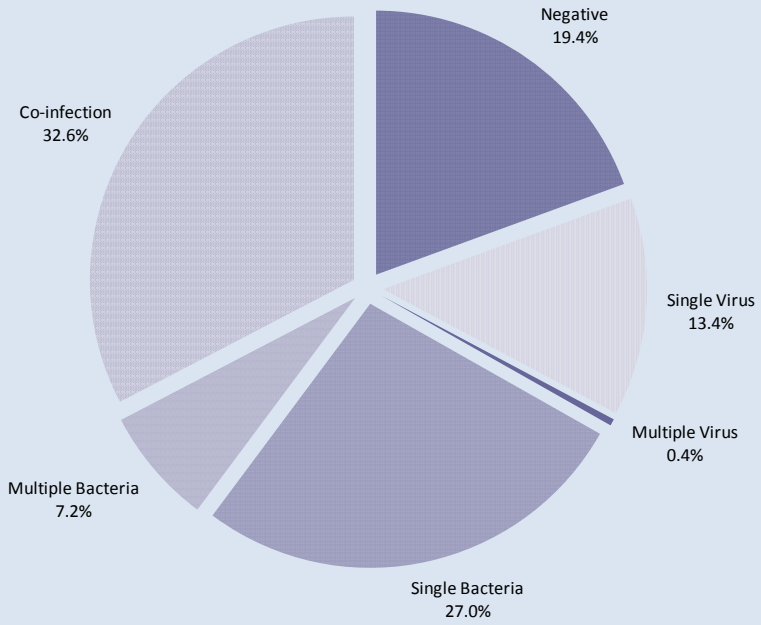
In total 585/726 (80.6%) were positive for a respiratory pathogen; no pathogen was identified in 141/726 (19.4%) of NPA samples. The aetiological agents identified among paediatric patients attending the Norfolk and Norwich University Hospital with symptoms of ARTI are shown in Figure 4.4. Of those paediatric patients for whom a pathogen was identified, 100/726 (13.8%) had a single or multiple viral infection, 248 (34.2%) had a single or multiple bacterial infection, and 237 (32.6%) had a mixed viral and bacterial infection (Figure 4.4).

4.4.3. Respiratory Viruses

A total of 303/726 (41.7%) NPA samples were positive for 1 or more respiratory viruses by a combination of virus culture, DFA and NOW® RSV Test between 31st October 2005 and 31st December 2008. HRSV was detected in 257/726 (35.4%) NPA samples; other viruses included rhinovirus (15/726; 2.1%); PIV (14/726; 1.9%); influenza virus type A (9/726; 1.2%), influenza virus type B (3/726; 0.4%); adenovirus (2/726; 0.3%), enterovirus (2/726; 0.3%) and a co-infection between adenovirus and enterovirus (1/726, 0.14%). HMPV was detected

retrospectively by real-time RT-PCR as the sole viral aetiologic agent in an additional 34/726 (4.7%) NPA samples that were previously identified as negative for respiratory viruses by a combination of virus culture, DFA, NOW® RSV Test and as co-pathogen with HRSV (3/726; 0.4%) and rhinovirus (1/726; 0.14%).

Figure 4.4. Aetiologial agents identified in nasopharyngeal aspirate samples collected from children ≤18 years attending the Norfolk and Norwich University Hospital with symptoms of acute respiratory tract infection between 31st October 2005 and 31st December 2008.



4.4.4. Respiratory Bacteria

A total of 1 or more respiratory bacteria were isolated from 485/726 (66.8%) NPA samples by bacterial culture. *S. pneumoniae* was isolated from 196/726 (27.0%) NPA samples and was the most common target bacterial pathogen isolated followed by *H. influenzae* (186/726; 25.6%), *S. aureus* (96/726; 13.2%) and *M. catarrhalis* (59/726; 8.1%). A single bacterial pathogen was isolated from 376 (51.8%) NPA samples while NPA samples with 2 or more bacteria was less common (109, 15%). *S. pneumoniae* and *H. influenzae* were the most common pairing in NPA samples with mixed bacterial aetiology (9.2%). No significant bacterial pathogen was isolated from 191 (26.3%) NPA samples and bacterial growth was absent from 50 (6.9%) samples.

4.4.5. Viral and Bacterial Co-infections

A total of 237/726 (32.6%) NPA samples were co-infected with respiratory viruses and 1 or more concomitant pathogenic bacteria. Bacterial and viral co-infections were more numerous than viral mono- and co-infections (13.8%) but less common than RTI caused by bacteria alone (34.2%). Multi-viral and bacterial co-infections were detected in 2 (0.28%) NPA samples; HMPV was detected as a co-pathogen with HRSV, *S. pneumoniae* and *H. influenzae* (1, 0.14%) and rhinovirus and *S. pneumoniae* (1, 0.14%). Concurrent infections with one or more respiratory bacteria and RSV or HMPV were identified in 180 (24.8%) and 27 (3.7%) NPA samples, respectively. *S. pneumoniae* (14.0%) was the most frequent bacterial pathogen occurring in combination with HRSV in NPA samples, followed by *H. influenzae* (9.4%), *S. aureus* (4.0%), and *M. catarrhalis* (2.8%). In contrast, *H. influenzae* (2.6%) succeeded *S. pneumoniae* (2.1%) as the dominant bacterial co-pathogen in paediatric patients with concurrent HMPV infection. However, *S. pneumoniae* and *H. influenzae* occurred as the principal union in NPA samples harbouring more than 1 bacterial pathogen and HRSV (4.0%) or HMPV (1.0%) as the viral co-pathogen. Concurrent infections with one or more respiratory bacteria and HRSV or HMPV was not significant (Mann-Whitney *U* test, p = 0.188) (Table 4.1).

Table 4.1. Comparison of the number of bacterial pathogens isolated by bacterial culture from nasopharyngeal aspirate samples collected between 31st October 2005 and 31st December 2008 from paediatric patients with concurrent human respiratory syncytial virus or human metapneumovirus infection.

Number of associated bacterial pathogens	No. positive NPA samples (%)	
	HMPV	HRSV
0	9 (23.7)	76 (29.6)
1	18 (47.4)	135 (52.5)
2	11 (28.9)	41 (16.0)
3	0 (0.0)	5 (1.9)
TOTAL	38 (100)	257 (100)

Abbreviations: NPA, nasopharyngeal aspirate; HRSV, human respiratory syncytial virus; HMPV, human metapneumovirus.

The significance of the association between HMPV and bacterial pathogens found in NPA samples was determined using Spearman's Rank Correlation Test. The presence of *H. influenzae* was positively correlated with the presence of HMPV (Spearman correlation coefficient = 0.131; p = 3.91E-04). However, no direct correlation was evident between the presence of *S. pneumoniae* and the presence of HMPV (Spearman correlation coefficient =

0.066; *p* = 0.075). In contrast, the presence of HRSV was not correlated with the presence of *H. influenzae (*Spearman correlation coefficient = 0.014; *p* = 0.702*)* but strongly correlated with the presence *S. pneumoniae* (Spearman correlation coefficient = 0.212; *p* = 8.53E-09*).* No association was substantiated between HRSV or HMPV and other concurrent bacterial pathogens identified in NPA samples. Logistic regression analysis was performed on viral and bacterial co-infections that included HMPV as the viral co-pathogen and *H. influenzae and S. pneumoniae*. Interestingly, only *H. influenzae* remained independently correlated with HMPV (Table 4.2)

Table 4.2. Associations between human respiratory syncytial virus or human metapneumovirus infection and concurrent bacterial pathogens identified in nasopharyngeal aspirate samples collected from paediatric patients between 31st October 2005 and 31st December 2008.

Bacterial pathogen	Total no. bacterial isolates	Total no. mixed virus-bacteria infections (spearman correlation coefficient, *p* value)	
		HMPV	HRSV
Streptococcus pneumoniae	196	15 (0.066, 0.075)	102 (0.212, 8.53E-9)
Haemophilus influenzae	186	19 (0.131*, 3.91E-4)	68 (0.014, 0.702)
Moraxella catarrhalis	59	3 (-0.002, 0.957)	20 (-0.009, 0.802)
Staphylococcus aureus	96	1 (-0.073, 0.048)	29 (-0.042, 0.254)
Coliform sp.	25	1 (-0.010, 0.778)	3 (-0.092, 0.013)

Probabilities of association by chance (*p*-values) are shown with figures indicating significant correlations at the 0.05 (*) level higlighted in red. Abbreviations: HRSV, human respiratory syncytial virus; HMPV, human metapneumovirus.

4.5. Discussion

Viral potentiation of bacterial superinfection of the respiratory tract due to the synergism between bacteria and viruses is well documented (Ampofo *et al*., 2008; Creer *et al*., 2006; Don *et al*., 2005; Finelli *et al*., 2008; Hamano-Hasegawa *et al*., 2008; Juvén *et al*., 2000; Lehtinen *et al*., 2006; McCullers and Bartmess 2003; O'Brien *et al*., 2000; Thorburn *et al*., 2006. However, the discovery of new respiratory viruses has commanded the continued surveillance of concomitant bacterial and viral respiratory tract infections in order to understand the nature of that synergism that predisposes to a protracted bacterial infection (Beder *et al*., 2009; Madhi *et al*., 2006; Kukavica-Ibrulj *et al*., 2009; Wolf *et al*., 2009). Evidence to support the importance of HMPV in the pathogenesis of LRTI suggests that preceding infection with HMPV predisposes the host to subsequent secondary bacterial infections (Ampofo *et al*., 2008; Kukavica-Ibrulj *et al*., 2009; Madhi *et al*., 2006; Wolf *et al*.,

2009). While these studies provide evidence to support the role of HMPV in the development of invasive pneumococcal pneumonia, evidence is thus so far lacking to form a complete picture of the relationship between HMPV and other bacteria that are habitually implicated in mixed viral-bacterial respiratory tract infections including *H. influenzae*, *M. catarrhalis*, *S. aureus*, and *S. pyogenes*. The aim of this observational study, based on the retrospective investigation of frozen, archived NPA samples, was to determine the frequency of bacterial and viral co-infections in children ≤18 years attending the Norfolk and Norwich University Hospital between 31st October 2005 and 31st December 2008 with symptoms of ARTI and provide evidence to support a potential role for other commensal flora of the nasopharynx in co-infections with HMPV.

A direct comparison of prevalence of one virus with other viruses requires a measure of caution when the diagnosis of viral disease is undertaken by different methods (Williams *et al*., 2004). It is widely reported that utilisation of molecular methodology for the diagnosis of respiratory viral infections offers greater sensitivity with a resultant increase in the detection rate of respiratory viral pathogens in comparison to traditional diagnostic methods (Balada-Llasat *et al.,* 2010; Coiras *et al*., 2004; Gadsby *et al*., 2010; Kehl *et al*., 2001; Kuypers *et al*., 2006; 2009; Marshall *et al*., 2007; Nolte *et al*., 2007; Templeton *et al*., 2004; Weinberg *et al*., 2004). Nevertheless, this increase in sensitivity is not universal for all viral respiratory pathogens (Balada-Llasat *et al.,* 2010; Gröndahl *et al*., 1999; Marshall *et al*., 2007; Puppe *et al*., 2004). Indeed, the increase in the detection rate of HRSV achieved by molecular methodology in comparison to traditional methods of virological diagnosis is less pronounced than for other viruses (Kuypers *et al*., 2006; LeGoff *et al*., 2008; Nolte *et al*., 2007). Hence, it is possible to attempt to establish the frequency of these viruses either alone or in combination with other bacteria that are habitually implicated in mixed viral-bacterial RTI in order to understand better the microbial community in children with ARTI. While the absence of molecular methodology for the detection of viruses other than HMPV is offered as a limitation of the present study, it is possible to find similarities with other studies regarding the epidemiology of RTI in hospitalised children. Bacteria and viruses accounted for 66.8% and 46.4% of infections, respectively, within this cohort. In an earlier study, bacteria and viruses accounted for 60% and 45% of infections, respectively (Michelow *et al*., 2004). Most significant was the finding that bacterial and viral co-infections accounted for 32.6% of infections. This suggests that co-detection of pathogens is the norm, not the exception, among acute-phase respiratory samples (Brunstein *et al*., 2008), which once again raises the important question of whether sequential or concurrent viral and bacterial

infections have a synergistic impact on the evolution of disease in children (Michelow *et al*., 2004). At present, this question remains unresolved.

HMPV was identified in 38 (5.2%) NPA samples, which in conjunction with other studies suggests that this virus causes respiratory tract infection in children at a relatively high frequency (Williams *et al*., 2004). Also comparable to earlier studies was the finding of male predominance in subjects with HMPV infection (Chan *et al*., 2007; Esper *et al*., 2004; Ljubin-Sternak *et al*., 2008; McAdam *et al*., 2004; Morrow *et al*., 2006; Peiris *et al*., 2003; Samransamruajkit *et al*., 2006; Williams *et al*., 2004). However, this trend is not universal (Boivin *et al*., 2003; Pizzorno *et al*., 2010). It is also equally plausible that the predilection for male gender is a reflection of the higher proportion of ARI hospitalisations in males. Nevertheless, male gender is associated with increased risk of LRTI (Simoes, 2003). Respiratory tract infection attributable to HMPV and HRSV occurred predominantly in the first year of life. In agreement with other studies, children with HMPV-associated RTI were older in comparison to children with HRSV-associated RTI (Baer *et al*., 2008; Camps *et al*., 2008; Morrow *et al*., 2006; Mullins *et al*., 2004; Peiris *et al*., 2003; Wolf *et al*., 2006). The difference could be due to longer lasting maternal immunity to HMPV compared with HRSV, or perhaps the pathogenesis of HMPV disease favours older children. More research is needed to answer these questions (Mullins *et al*., 2004).

Respiratory infections attributed to HMPV coincided with the annual HRSV epidemic in part or entirely with peak HMPV activity mirroring or following peak HRSV activity as reported elsewhere (Aberle *et al*., 2008). However, differences in the seasonal incidence of these viruses exist within this paediatric cohort. In general, HRSV infections occurred mainly in autumn and winter while HMPV infections occurred in winter and spring. This pattern of seasonality is widely reported in conjunction with alternating years of high and low incidence (Agapov *et al*., 2006; Baer *et al*., 2008; Esper *et al*., 2004; Kaida *et al*., 2006; Maggi *et al*., 2003; McAdam *et al*., 2004; Mullins *et al*., 2004; Oliveira *et al*., 2008; Robinson *et al*., 2005) although within the present study this seasonal rhythm of HMPV activity is only evident during 2005/2006 and 2007/2008.

HMPV was identified as the sole etiological agent in 7 (1%) NPA samples but was more commonly associated with 1 or more concomitant respiratory bacteria (27; 3.7%). Co-infections with HMPV and other respiratory viruses (2; 0.28%) and other respiratory viruses and bacteria (2; 0.28%) were rare. These results suggest the direct involvement of HMPV in

the development of bacterial-viral co-infections and reiterate that the pathogenesis of HMPV-associated ARTI frequently involves bacterial co-infection (Madhi *et al*., 2006). Asymptomatic nasopharyngeal carriage of *S*. *pneumoniae*, *H*. *influenzae*, *M*. *catarrhalis*, and *S*. *aureus* is common in children (Pettigrew *et al*., 2008). However, these bacteria also remain an important cause of LRTI in children and so their presence in respiratory specimens can be difficult to interpret (Wolf *et al*., 2007). Bacterial growth from NPA samples is not indicative of infection in the lower respiratory tract (Korppi *et al*., 1992). Yet, it is unjustified to exclude the contribution of these potential bacterial pathogens to the development of RTI and in particular in the presence of concomitant viral infections that predispose to bacterial superinfections of the respiratory tract (Bakaletz, 1995). Long-standing epidemiological evidence supports that the pairing of certain infectious agents is not a random association but a synergistic or sequential enterprise between complementary partners that leads to modification of the outcome of an infection compared to the course of infections caused by single pathogen (Bakaletz, 2004; Degré, 1986). Data analysis showed that the presence of *H. influenzae* was positively correlated with the presence of HMPV (Spearman correlation coefficient = 0.131; p = 3.91E-04). However, no direct correlation was evident between the presence of *S. pneumoniae* and HMPV (Spearman correlation coefficient = 0.066; p = 0.075)**.** In contrast, the presence of *S. pneumoniae* was strongly correlated with the presence HRSV (Spearman correlation coefficient = 0.212; p = 8.53E-09). This is consistent with reported interactions between this viral-bacterial pairing (Hament *et al*., 2005) and epidemiologic observations of increased incidence of invasive disease when S*. pneumoniae* and RSV co-circulate (Talbot *et al*., 2005).

Failure to establish a direct link between HMPV and *S. pneumoniae* within this study may reflect epidemiological differences in the UK compared to elsewhere. Madhi *et al*. (2006) demonstrated that vaccination with PVC9 reduced the incidence of HMPV-associated LRTI among a cohort of children in South Africa in a study designed to determine the efficacy of the vaccine to prevent invasive pneumococcal disease (IPD) and radiographically confirmed pneumonia. In addition, the observations from a cohort study conducted in the Netherlands revealed that frequent nasopharngeal carriage of *S. pneumoniae*, but not *H*. *influenzae*, *M. catarrhalis* or *S. aureus*, was associated with increased seroconversion rates of infants to HMPV at the age of 2 years. However, vaccination against *S. pneumoniae* was not part of the National Immunisation Programme at the time of serum collection (Verkaik *et al*., 2011). A 7-valent pneumococcal conjugate vaccine (PCV7, Prevenar™) providing protection against serotypes 4, 6B, 9V, 14, 18C, 19F and, 23F was introduced into the routine childhood

immunisation programme in England and Wales in September 2006 (Kaye *et al*., 2009) to reduce the burden of IPD (DoH, 2006). Current data for England and Wales show a dramatic 41% decrease in the number of IPD cases in children under 5 years of age between epidemiological years 2005–2006 (797 cases) and 2007–2008 (470 cases) following the introduction of the vaccine (Kaye *et al*., 2009). Moreover, IPD attributed to serotypes present in PCV7 which, accounted for 70% of cases of IPD among this age group in epidemiological year 2005/2006 decreased to 24% in 2007/2008 and 10% in 2008/2009 (Kaye *et al*., 2009; Pichon *et al*., 2009). In addition to the decrease in IPD in the vaccinated population, herd immunity to vaccine serotypes has been induced in the UK population as an indirect effect of infant PCV7 immunisation (Gladstone *et al*., 2011) with decreases in all vaccine serotypes in all age groups (Miller *et al*., 2011). Given the marked reduction in rate of cumulative increase of cases of IPD caused by the vaccine serotypes since vaccine introduction (Kaye *et al*., 2009), the lack of correlation between HMPV and *S. pneumoniae* in the present study may reflect the reduction in the nasopharyngeal carriage of vaccine serotypes of *S. pneumoniae* that has arisen through widespread vaccination with PCV7.

The introduction of PCV7 into the childhood immunisation schedule in the United States has directed a marked decline in vaccine serotypes carried in the nasopharynx of young children, with a coincident rise in the prevalence of non-vaccine serotypes (Pelton *et al*., 2004). Interestingly, a recent study carried out in Utah demonstrated that preceding respiratory tract infection with HMPV was associated with increases in IPD in children attributable to non-vaccine serotypes (Ampofo *et al*., 2008). Hence, the postulated association between HMPV and pneumococcal infection may present an emerging health threat in the UK since an increase in the incidence of IPD caused by pneumococci of non-vaccine serotypes, a phenomenon known as 'serotype replacement' has occurred in the UK (Gladstone *et al*., 2011).

It is clear that widespread PC7 vaccination is having a significant impact on the epidemiology of pneumococcal disease among children as well as adults. The reduction in nasopharyngeal colonisation by vaccine serotypes has placed non-vaccine serotypes in a prime position to fill the specific niche vacated by their counterparts in an environment under vaccine pressure (Gladstone *et al*., 2011). However, it seems the changes imposed on the colonisation of the nasopharynx by *S. pneumoniae* following the introduction of the PCV7 vaccine are not limited to this specific bacterial species. Indeed, the reduction in vaccine serotypes has exposed a niche for enhanced colonisation and disease caused by other commensals of the

nasopharynx (van Gils *et al*., 2011). Several studies have demonstrated an inverse relationship between *S*. *pneumoniae* carriage, and specifically vaccine serotypes, and *S*. *aureus* carriage in children (Bogaert *et al*., 2004; Regev-Yochay *et al*., 2004; Madhi *et al*., 2007b; McNally *et al*., 2006; Pettigrew *et al*., 2008). The implication of this relationship is that *S. pneumoniae* carriage provides protection against *S. aureus* carriage (Brogden *et al*., 2005). This has raised concerns that the reduction in nasopharyngeal carriage of vaccine-type *S. pneumoniae* could result in a concomitant increase in carriage rates of *S. aureus* including methicillin-resistant *S. aureus* and an associated increase in *Staphylococcus*-related infections (Brogden *et al.,* 2005; Singh, 2007).

Recognition of this previously unrecognised interaction between *S. pneumoniae* and *S. aureus* has raised awareness of the possible effects of PCV-7 on the natural balance of other commensal flora of the nasopharynx. Indeed, a demonstrable shift in frequency of causative organisms recovered from children with AOM (Casey *et al*., 2004; Block *et al*., 2004) and acute bacterial maxillary sinusitis (Brook *et al*., 2007) was observed with the replacement of *S. pneumoniae* with *H. influenzae* as the dominant pathogen (Brook *et al*., 2007; Casey *et al*., 2004) following the introduction of universal immunisation with PCV7 in the United States. The reduction in the nasopharyngeal carriage of vaccine serotypes of *S. pneumoniae* that has arisen through widespread vaccination with PCV7 in England and Wales may offer *H. influenzae* a competitive advantage to colonise the vacant nasopharyngeal niche unimpeded by *S. pneumoniae*.

Our understanding of the competitive interactions between *S. pneumoniae and H. influenzae* are limited. Competition between *H. influenzae* and *S. pneumoniae* for mucosal surfaces of the respiratory tract in a murine model induced the recruitment and activation of innate immune responses by *H. influenzae*, leading to the effective clearance of *S. pneumoniae* (Lysenko *et al*., 2005). *In vitro* studies also demonstrate competition between *H. influenzae* and *S. pneumoniae* but predict that *S. pneumoniae* should inhibit the growth of *H. influenzae* (Pettigrew *et al*., 2008). *In vitro* co-culture experiments conducted to examine the interactions of the co-inhabitants of the heavily colonised mucosal surface of the human upper respiratory tract suggest that elevated hydrogen peroxide production may provide a means by which *S. pneumoniae* is able to inhibit *H. influenzae* in the aerobic environment of the upper respiratory tract (Pericone *et al*., 2000). In addition, *S. pneumoniae* expresses neuraminidase which desialylates the lipopolysaccharide of *H. influenzae* (Shakhnovich *et al*., 2002), potentially giving *S. pneumoniae* a competitive advantage by increasing the

susceptibility of *H. influenzae* to complement-mediated clearance (Pettigrew *et al*., 2008). While *in vivo* and *in vitro* studies show interesting and sometimes contradictory results (Murphy *et al*., 2009), such studies reiterate that the pathophysiology associated with these organisms is complex. It is plausible that the reduction in the nasopharyngeal carriage of vaccine serotypes of *S. pneumoniae* that has arisen through widespread vaccination with PCV7 in the England and Wales has offered *H. influenzae* a competitive advantage to colonise the nasopharyngeal niche whichever model of the polymicrobial competition is considered.

CHAPTER FIVE

5. Frequency of multiple pathogens in acute respiratory infections

5.1. Introduction

Virus isolation by cell culture using a variety of permissive cell lines and DFA staining with monoclonal antibodies are two of the most commonly used techniques for detecting respiratory viruses in routine diagnostic laboratories (Syrmis *et al*., 2004). Virus culture has long served as the "gold standard" for laboratory diagnosis of respiratory viruses (Leland and Ginocchio, 2007). However, it is often too slow to be an optimal diagnostic technique (Englund, 2001) and therefore, its clinical value is limited (Templeton *et al*., 2004). Antigen detection via enzyme immunoassays or immunofluorescence microscopy, are widely used in the establishment of viral aetiologies of respiratory tract infection (Carroll, 2002) but suffer greatly from lack of sensitivity and specificity, especially during times of low prevalence or in special populations, such as the immunocompromised or the elderly (Henrickson, 2004). While the combination of both these techniques can provide an increase in the number of positive results, a significant proportion of specimens remain negative despite clinical suspicion of viral infection (Syrmis *et al*., 2004). A shortage in the necessary skills and resources to culture most viruses also represents a foreseeable challenge to many clinical virology laboratories. Notwithstanding advancements made in these traditional methods or the continued importance of virus culture and related techniques in special situations including the identification of unknown infectious agents, their limitations have led to unmet needs within the diagnostic laboratory being addressed by new tools, most notably nucleic acid amplification techniques (Krunic *et al*., 2011; Olofsson *et al.,* 2011). Many molecular diagnostic methods are now widely accepted as the gold standard for the detection of respiratory viruses owing to resolution of intrinsic limitations associated with traditional diagnostic techniques through the coalescence of enhanced sensitivity, specificity, wide linear dynamic range, and speed (Ellis and Curran, 2010; Beck and Henrickson, 2010; Henrickson, 2004; Syrmis *et al*., 2004). However, it is important to recognise that the use of culture-independent techniques are based on the same diagnostic processes as traditional diagnostic methods, which assume a single agent aetiology and cease with the detection of the first relevant infectious agent (Brunstein *et al*., 2008; Rogers *et al*., 2010). This approach has often hampered the translatable clinical benefits achieved by the diagnostic use of PCR (Rogers *et al*., 2010).

The arrival of multiplexed molecular methods has introduced a significant new element to the diagnosis of infectious disease, which has proved invaluable in the context of outbreak

management, improvements to patient treatment, and the efficient use of precious antibiotics and antiviral drugs (Lin *et al*., 2007; Mahony, 2007). However, the biggest transformation has arisen within the clinical virology setting, particularly in relation to the diagnosis of respiratory virus infection. Multiplexed PCR has provided a rapid, sensitive, specific, and cost effective alternative to traditional methods and monospecific PCR since it permits the simultaneous detection and identification of multiple infectious agents in a single reaction (Templeton *et al*., 2004). Furthermore, these methods have enabled laboratories to meet the diagnostic challenge associated with the emergence of new respiratory viruses, which have proven difficult to detect by traditional methods (Krunic *et al*., 2011). A variety of multiplexed molecular methods are described including conventional multiplex RT-PCR (Bellau-Pujol et al., 2005; Coiras *et al*., 2004; Gröndahl et al., 1999; Osiowy, 1998), multiplex real-time RT-PCR (Gunson *et al*., 2005; Kuypers *et al*., 2006; Templeton *et al*., 2004), as well as an increasing number of novel multiplex PCR systems (Brunstein *et al*., 2008; Kim et al., 2009; Li *et al*., 2007; Mahony *et al*., 2007; Nolte *et al.,* 2007; Poritz *et al*., 2011). The increased sensitivity and expanded diagnostic arsenal that has accompanied these multiplexed molecular methods (Olofsson *et al.,* 2011) has also revealed that respiratory virus infection with multiple agents is a frequent occurrence (Brunstein *et al*., 2008; Greer *et al*., 2009; Peng *et al*., 2009). The idea that some infectious diseases involve multiple pathogenic players is not new (Brogden and Guthmiller, 2003). Indeed, the mixed microbial nature of diseases of humans and animals was recognised early in the 20th century (Bakaletz, 2004). While these infections—variously called co-infections or complex infections, complicated infections, dual infections, mixed infections, secondary infections, synergistic infections, or polymicrobial infections—are more commonly accepted as occurring in respiratory infections than in many other clinical settings, diagnostic bias towards single pathogen detection and subsequent treatment is still prevalent (Brogden and Guthmiller, 2003; Brunstein *et al*., 2008). However, respiratory infection with multiple respiratory viruses is found in 5.2 to 35% of patients (Bellau-Pujol *et al*., 2005; Calvo *et al*., 2008; Cilla *et al*., 2008 ; Coiras *et al*., 2004; Frobert *et al*., 2011; Gadsby *et al*., 2010; Mahony *et al*., 2007) depending on the number of viruses targeted in the diagnostic panel. Co-infections with HMPV as well as HRSV, HRV, and human bocavirus (HBoV) are often reported (Brunstein *et al*., 2008; García-García *et al*., 2006a; Maggi *et al*., 2003; Richard *et al*., 2008; Schildgen *et al*., 2008). At present, the clinical significance of co-infection with these viruses remains controversial as evidence to support an association between co-infection and disease severity (Calvo *et al*., 2008; Foulongne *et al*., 2006; Greensill *et al*., 2003; König *et al*., 2004; Richard *et al*., 2008; Semple *et al*., 2005) is confounded by reports that contest an

association (Greer *et al*., 2009; Söderlund-Venermo *et al*., 2009; van Woensel *et al*., 2006; Wilkesmann *et al*., 2006; Wolf *et al*., 2006). A resolution to the conundrum may lie in the ability to determine the quantity of co-infecting infectious agents in order to distinguish a true aetiological agent from an innocent bystander (Olofsson *et al.,* 2011). Indeed, a broad diagnostic panel offers additional diagnostic value. First, the validity of a negative result is improved when many infectious agents are targeted and second, the quantitative component of the test, as measured by C_T values, enhances the interpretation of a positive result, particularly when several agents are detected and the C_T values of those agents can be compared (Olofsson *et al.,* 2011).

The recent advancement in molecular techniques has enhanced the identification and characterisation of the vast microbial diversity colonising the human body (Brogden *et al*., 2005) and may also clarify which virus is the true aetiological agent but also the passenger virus in these infections (Olofsson *et al.,* 2011). Whilst many of these techniques have not yet reached laboratories in a scale to assist clinicians in practice (Brogden *et al*., 2005), multiplexed methods, which allow the identification of even minor populations of co-infecting viruses, are beginning to provide a more accurate representation of the true pathogen spectrum present in acute-phase respiratory specimens (Brunstein *et al***.,** 2008; Olofsson *et al.,* 2011), particularly in relation to polyviral respiratory infections, which are poorly understood *in vivo* (Ruuskanen *et al*., 2011). Perhaps, in future this will extend to bacterial pathogens, which are generally studied individually, although in their natural environment they often co-exist or compete with multiple microbial species (Murphy *et al*., 2009). Highly evolved relationships exist between microbes and it is important to understand these relationships, especially in settings when flora is manipulated with the possibility of affecting other pathogens (Murphy *et al*., 2009). The first detailed studies of multipathogen interactions demonstrate that viral co-infections are not random; significant correlations for the occurrence of certain viral agents exist (Brunstein *et al*., 2008). In contrast to bacteria, the role of viruses has historically been as the instigators of infection rather than as colonising commensals. However, a new hypothesis suggests that the presence of viruses in the respiratory tract may not always be as a cause of infection (Greer *et al*., 2009). Indeed, Greer *et al*., (2009) suggest that acquisition of HRV infection can protect its host from infection by other, often more cytopathic, viruses. Other evidence suggests that most interactions between pathogens with resultant co-suppression are reciprocal and occur between single-stranded RNA viruses inferring the activation of non-specific antiviral functions by the first infecting agent; inhibition of infection initiation by a

second RNA virus in the face of multiple activated antiviral responses would be a plausible outcome (Brunstein *et al*., 2008). While this phenomenon is new to respiratory viruses perhaps relationships long regarded as 'common lore' (Bakaletz, 1995) may be assuaged in consideration of new paradigms generated by molecular techniques regarding the role of viruses in the development of respiratory tract infection.

5.2. Aims

The current chapter commences with an evaluation of the xTAG® RVP test, a proprietary test that simultaneously detects up to 20 distinct respiratory virus targets in parallel with traditional diagnostic methods. This evaluation was performed in order to determine the frequency of emerging respiratory viruses, which have proven difficult to detect by traditional methods as well as well-established respiratory viruses, since it is widely reported the contribution of these viruses to respiratory infection is under-represented by traditional diagnostic methods in comparison to nucleic acid amplification techniques. The outcome of this initial aim provided the basis to construct a plan of change that comprised a retrospective evaluation of an in-house multiplex real-time RT-PCR assay for the diagnosis of an extended panel of respiratory viruses with a view to introducing this non-commercial assay into routine service provision.

In the second part of this chapter, the interactions among pathogens in multipathogen infections in hospitalised children with ARTI were examined with particular reference to HMPV. Recent advances in methods to diagnose respiratory infections have provided evidence that simultaneous infection with multiple respiratory viruses is a frequent occurrence in children admitted with ARTI. However, few studies have examined the interactions of well-established viruses with emerging respiratory viruses or respiratory bacteria.

5.3. Materials and Methods

5.3.1. Overview of study design

This study was based on the investigation of 201 respiratory specimens, which were submitted to the Specialist Virology Centre, Norfolk and Norwich University Hospital during the period October 2007 to December 2008. Approximately 16 specimens were selected at random each month for retrospective testing by the xTAG® RVP test at Lab21 Ltd, a commercial healthcare diagnostics company based in Cambridge, UK. Frozen aliquots of

xTAG® RVP tested samples were subsequently retrieved from storage at -70°C, and tested in a retrospective evaluation of an in-house multiplex real-time RT-PCR assay for the diagnosis of 12 different respiratory viruses and subtypes.

Comprehensive virological and bacteriological screening was available for 140 NPA samples collected from children ≤18 years attending the Norfolk and Norwich University Hospital with symptoms of acute respiratory tract infection.

5.3.2. Routine Diagnosis of Respiratory Infection

All samples were examined by virus culture, DFA (IMAGEN™, Oxoid Ltd, Hampshire, UK), and NOW® RSV Test (Binax, Inc., ME, USA) for influenza virus types A and B, HRSV, PIV types 1-3, HRSV, adenovirus, and *Chlamydia* sp and cultured on appropriate bacteriological media for the isolation of target organisms including *H. influenzae*, *M. catarrhalis*, *S. aureus*, *S. pneumoniae* (Section 2.4). A new direct immunofluorescence test for the rapid detection and identification of HMPV (IMAGEN™, Oxoid Ltd, Hampshire, UK) in clinical specimens was in routine usage throughout the evaluation the xTAG® RVP test.

5.3.3. Nucleic acid extraction

Total nucleic acid was extracted from NPA samples preserved in VTM using the COBAS® AmpliPrep TNAI Kit (Roche Diagnostics Ltd, Burgess Hill, UK) on the COBAS® AmpliPrep Instrument (Roche Diagnostics Ltd, Burgess Hill, UK), according to manufacturer's instructions (Section 2.6.4.4). A positive control for each viral target and a negative control consisting of nuclease-free water were included in each run.

5.3.4. Selection of primers and probes

The real-time quadriplex PCR for the detection of influenza (HPA, 2006) (Appendix III) was combined with primer and probe sequences designed according to Dr Martin Curran, CMPHL, Addenbrooke's Hospital, Cambridge to facilitate the simultaneous detection and identification of an extended panel of human respiratory virus types and subtypes, including all generic influenza virus A subtypes (H1-H15) and specifically H5 subtypes; influenza virus type B; HPIV types 1 to 4; HRSV; HMPV; adenovirus; HRV, and human enterovirus. The primer and probe sequences for the 12 different respiratory virus types and subtypes were divided into three multiplex real-time RT-PCR assays with up to 4 viral targets detected in a single tube (Appendix IV). The viruses and subtypes within each multiplex real-time RT-PCR

assay were discriminated using TaqMan® or TaqMan® MGB™ probes labelled with a fluorescent reporter dyes 6-FAM, VIC®, a proprietary dye only available from Applied Biosystems, ROX or cyanine (CY)-5 at the 3' end and a compatible non-fluorescent Black Hole Quencher molecule at the 5' end. The real-time quadriplex RT-PCR assay for the detection of influenza viruses formed the first panel. The second and third panel comprised HRSV, HPIV types 1 and 3, and adenovirus and third panel HPIV types 2 and 4, HMPV, HRV and enterovirus, respectively. The third panel was optimised already for HMPV detection and so was not altered under the proviso that the existing sequences compared favourably with the primer and probe sequences described by Maertzdorf *et al*., (2006). The primer and probe sequences that comprised each panel and the gene targets are shown in Appendix III.

5.3.5. Detection of respiratory viruses using three multiplex RT-PCR assays

A working reaction mix was prepared for each respiratory virus panel by combining the SuperScript™ III Platinum® One-Step qRT-PCR System (Invitrogen Ltd, Paisley, UK) (Section 2.6.6.2) in a final reaction volume of 25 µl with the appropriate combination of primers and TaqMan® or TaqMan® MGB™ probes at the concentrations shown in Table 3.3 in section 3.3.4) for panel I, and Table 5.1 for panels II and III with nuclease-free water (Bioline Ltd London , UK) to a volume of 20 µl. Finally, 5 µl of viral RNA extract was added exclusive of the NTC. Instead, 5 µl of nuclease-free water was added. RT-PCR was performed on the Rotor-Gene 6000 real-time system (Corbett Research Ltd, Cambridge, UK) using the thermal cycling parameters (Section 2.6.6.2) acquiring on the FAM, VIC, CY-5 and ROX channels. Real-time measurements were taken at each cycle.

Table 5.1. Concentrations of oligonucleotides added to the reaction mixture in preparation of panel II and panel III of the respiratory multiplex real-time reverse-transcriptase polymerase chain reaction assay for the detection of an extended panel of respiratory viruses

Panel II Reagents	**Volume (µl) per 25 µl reaction**	**Stock Concentration**	**Final Concentration**
SuperScript III RT/Platinum® *Taq* Mix	12.5	-	-
2X Reaction Mix	0.8	2X	1X
Magnesium sulphate ($MgSO_4$)	1.5	50 mM	4.5 mM
HRSV forward primer	0.50	20 µM	400 nM
HRSV reverse primer	0.50	20 µM	400 nM
HRSV probe	0.20	10 µM	80 nM
HPIV 1 forward primer	0.50	20 µM	400 nM
HPIV 1 reverse primer	0.50	20 µM	400 nM
HPIV 3 forward primer	0.50	20 µM	400 nM
HPIV 3 reverse primer	0.50	20 µM	400 nM
HPIV 1&3 probe	0.20	10 µM	80 nM
ADENO forward primer	0.16	20 µM	128 nM
ADENO reverse primer	0.16	20 µM	128 nM
ADENO probe	0.10	10 µM	40 nM
Nuclease-free water	1.38	-	-
TOTAL VOLUME	20.00		

Panel III Reagents	**Volume (µl) per 25 µl reaction**	**Stock Concentration**	**Concentration (nM)**
SuperScript III RT/Platinum® *Taq* Mix	12.5		
2X Reaction Mix	0.8	2X	1X
Magnesium sulphate ($MgSO_4$)	1	50 mM	4 mM
EnV/hRV forward primer	0.50	10 µM	400 nM
EnV/hRV reverse primer	0.50	10 µM	400 nM
EnVprobe	0.30	5 µM	120 nM
hRV probe	0.30	20 µM	120 nM
hMPV forward primer	0.50	20 µM	400 nM
hMPV reverse primer	0.50	20 µM	400 nM
hMPV probe	0.30	10 µM	120 nM
HPIV 2 forward primer	0.25	20 µM	200 nM
HPIV 2 reverse primer	0.25	20 µM	200 nM
HPIV 2 probe	0.10	10 µM	40 nM
HPIV 4 forward primer	0.25	20 µM	200 nM
HPIV 4 reverse primer	0.25	20 µM	200 nM
HPIV 4 probe	0.10	10 µM	40 nM
Nuclease-free water	1.60	-	-
TOTAL VOLUME	20.00		

5.3.6. xTAG® Respiratory Virus Panel Test

The xTAG® RVP test is a multiplexed nucleic acid assay for the simultaneous detection and identification of 20 different human respiratory virus types and subtypes, including influenza virus type A as well as subtypes H1, H3, and H5; influenza virus type B; HPIV types 1 to 4; HRSV subtypes A and B; HMPV; adenovirus; rhinovirus; enterovirus; and HCoV OC43, HCoV 229E, HCoV NL63, HCoV HKU1, and SARS-CoV (Mahony *et al*., 2007). In addition, RNA bacteriophage MS2 serves as the internal positive control to monitor the effect of extraction and address the problems associated with the presence of PCR inhibitors while DNA bacteriophage lambda is used as an amplification and assay performance control (Pabbaraju *et al*., 2011). The xTAG® RVP test incorporates a coupled multiplex RT-PCR to generate specific amplicons followed by a multiplex Target-Specific Primer Extension (TSPE) (Merante *et al*., 2007). The target-specific primers are chimeric primers containing the target sequence juxtaposed to a proprietary Universal Tag sequence which allow for sorting on the Luminex® xMAP® platform (Merante *et al*., 2007). Viral nucleic acid was extracted from respiratory specimens and a multiplex RT-PCR was performed under optimised conditions in a single multiplex (16-plex) RT-PCR producing amplicons for each of the virus types/subtypes present in the specimens. The amplification products once generated were treated with Shrimp Alkaline Phosphatase (SAP) to inactivate unincorporated nucleotides and Exonuclease I (EXO) to degrade unutilised single stranded primers (Merante *et al*., 2007). The treated amplification products were subjected to multiplex TSPE reaction to detect viral targets present in the sample. In this step, each virus target was specifically hybridised to a Target-Specific Primer (TSP) possessing a unique DNA tag. A DNA polymerase extended perfectly formed complements and simultaneously incorporated biotin-dCTP into the extension product (Merante *et al*., 2007). After TSPE, the extension products were added directly to microwells containing an anti-tag coupled 21-bead array. The beads contained an anti-tag sequence unique to each specific viral target and were spectrally distinguishable from each other (Merante *et al*., 2007). A fluorescent reporter molecule, typically streptavidin-phycoerythrin, was hybridised to the biotin-labelled TPSE products. Each tagged primer hybridised only to its unique anti-tag complement; therefore, each bead represented a specific virus by virtue of the bead/anti-tag/tagged primer association. The beads were sorted and analysed by the Luminex® xMAP® platform (Merante *et al*., 2007). The platform contains two lasers: one identifies the fluorescent bead signature, and the other identifies the presence or absence of primer extension products through the phycoerythrin reporter molecule (Merante *et al*., 2007). The median fluorescent intensity (MFI) of each specific

bead population in the solution microarray is recorded. Results for viral targets are categorised as positive (MFI ≥ 300), negative (MFI < 150), or "no call." Assuming adequate performance of the internal control, a no-call result for a viral target is likely due to an MFI value in the equivocal zone (150 ≤ MFI < 300) (McCloskey *et al*., 2011).

5.3.7. Statistics

Characteristics and outcomes according to pathogen were compared using Spearman's rank correlation coefficient. Statistical calculations were performed with SPSS version 17.0 (SPSS Inc, Chicago, Illinois, USA). A *p* value of <0.05 was considered statistically significant.

5.4. Results

5.4.1. Characteristics of patients and specimens tested

From clinical respiratory specimens submitted to the Microbiology Department for routine microbiological investigations between October 2007 and December 2008, 201 were selected at random for retrospective testing by the xTAG® RVP test at Lab21 Ltd, a commercial healthcare diagnostics company based in Cambridge, UK. All samples were surplus to routine diagnostic requirements. Clinical specimens were predominantly from children ≤ 18 years (n= 154; 77% NPA samples) that attended the Norfolk and Norwich University Hospital with symptoms of acute respiratory illness. Other specimen types included 18 combined nose and throat swabs, 23 throat swabs, 1 sputum specimen, 2 BAL specimens, 2 lung aspirate specimens, and 1 endotracheal aspirate specimen. Seventeen combined nose and throat swabs and 9 throat swabs were collected from patients of all ages that presented with ILI or other acute respiratory illness to sentinel general practices that participated in the national virological influenza surveillance scheme during winter 2007/2008 and 2008/2009.

5.4.2. Performance of the xTAG® RVP test in relation to traditional diagnostic methods

The results of the xTAG® RVP test were analysed in parallel with results obtained during routine investigations for respiratory viruses by a combination of virus culture, DFA, and NOW® RSV Test. In total, a respiratory virus was identified in 151/201 (75%) specimens by the xTAG® RVP test compared with only 46/201 (23%) specimens by virus culture, DFA, and NOW® RSV Test. Concordance between the xTAG® RVP test and virus culture, DFA, and NOW® RSV Test was achieved for 94/201 (47%) specimens (Table 5.2). Respiratory viruses

common to the xTAG® RVP test and the virus culture, DFA, and BinaxNOW® RSV test panel including HMPV, influenza viruses type A and B, HRSV, HPIV types 1 to 4, and adenovirus were identified in 45/201 (22%) specimens. One specimen, which was HRSV positive by virus culture was not detected by the xTAG® RVP test. An additional 106 specimens were positive by the xTAG® RVP test. The combination of virus culture, DFA, and BinaxNOW® RSV test failed to diagnose mutual viruses in 42/106 (40%) specimens, while 64/106 (60%) specimens were negative for viruses not included in the current diagnostic panel offered by the combination of virus culture, DFA, and BinaxNOW® RSV test.

Table 5.2. Distribution of results obtained by the xTAG® respiratory virus panel test and a combination of virus culture, direct immunofluorescence and BinaxNOW® RSV Test for 201 respiratory specimens.

No specimens	Virus Culture/DFA/ NOW®RSV Test	xTAG® RVP test
45	Positive	Positive
49	Negative	Negative
1	Positive	Negative
106	Negative	Positive

Abbreviations: RVP, respiratory virus panel; DFA, direct immunofluorescence

The performance of the combination of virus culture, DFA, and BinaxNOW® RSV test relative to the xTAG® RVP test for diagnosis of respiratory virus infection is shown in Table 5.3 using the xTAG® RVP test as the gold standard. The overall sensitivity and specificity of the combination of virus culture, DFA, and BinaxNOW® RSV test in comparison to the xTAG® RVP test as the gold standard was 24.9% and 99.9%, respectively. Discrepancies between the xTAG® RVP test and the combination of virus culture, DFA, and BinaxNOW® RSV test were most prominent for enterovirus/rhinovirus, HRSV, and HMPV. Respiratory virus infection attributed to enterovirus/rhinovirus was diagnosed in an additional 83 respiratory specimens by the xTAG® RVP test while an extra 10 infections with HRSV and 14 infections with HMPV were identified by the xTAG® RVP test in comparison to the combination of virus culture, DFA, and BinaxNOW® RSV test (Table 5.3).

Table 5.3. Performance of the xTAG® respiratory virus panel test and a combination of virus culture, DFA, and BinaxNOW® RSV Test for diagnosis of respiratory virus infection in 201 respiratory specimens. The combined testing procedure was compared to the xTAG® RVP test as gold standard for viruses common to both tests.

Virus	CTP + xTAG +	CTP – xTAG +	CTP + xTAG –	CTP – xTAG –	Sensitivity (%)	Specificity (%)
EnV/HRV	7	83	0	111	7.80	100.0
HRSV	24	10	1	167	70.6	99.4
HPIV	5	7	0	189	41.7	100.0
HMPV	1	14	0	186	6.70	100.0
FA	3	5	0	193	37.5	100.0
FB	5	5	0	191	50.0	100.0
AdV	0	4	0	197	0.00	100.0

Abbreviations: EnV/HRV, human enterovirus/human rhinovirus, HRSV, human respiratory syncytial virus; HPIV, human parainfluenza virus; HMPV, human metapneumovirus; FA, influenza virus type A; FB, influenza virus type B; AdV, adenovirus; RVP, respiratory virus panel; CTP, combined testing procedure.

The results of the xTAG® RVP test revealed that multiple infections are frequent. In total, 125 (62%) specimens were positive for 1 respiratory virus, while 22 (11%) were positive for 2 respiratory viruses and 4 (2.0%) were positive for 3 respiratory viruses (Figure 5.1).

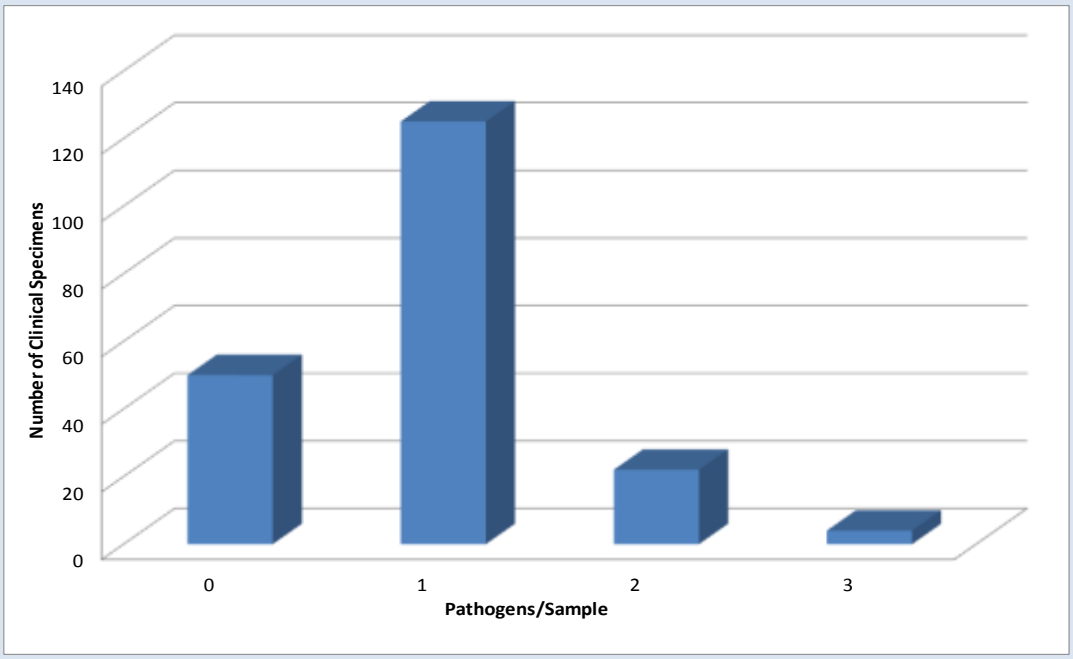


Figure 5.1. Prevalence of respiratory viruses detected by the xTAG® respiratory virus panel test in each clinical specimen.

Within specimens that yielded concordance between the xTAG® RVP test and the combination of virus culture, DFA, and BinaxNOW® RSV, co-infections with other respiratory viruses were found in 8/45 (18%) specimens by xTAG® RVP test; a second respiratory virus was found in 6 specimens and 2 specimens were co-infected with 2 further viral pathogens. Of the 106 specimens that were positive by the xTAG® RVP test only, a single pathogen was identified in 88/106 (83%) while co-infections with 2 or more respiratory viruses were found in 18/106 (17%) clinical specimens. The overall prevalence of viruses that were detected by the xTAG® RVP test in contrast to the routine diagnostic repertoire is shown in Figure 5.2.

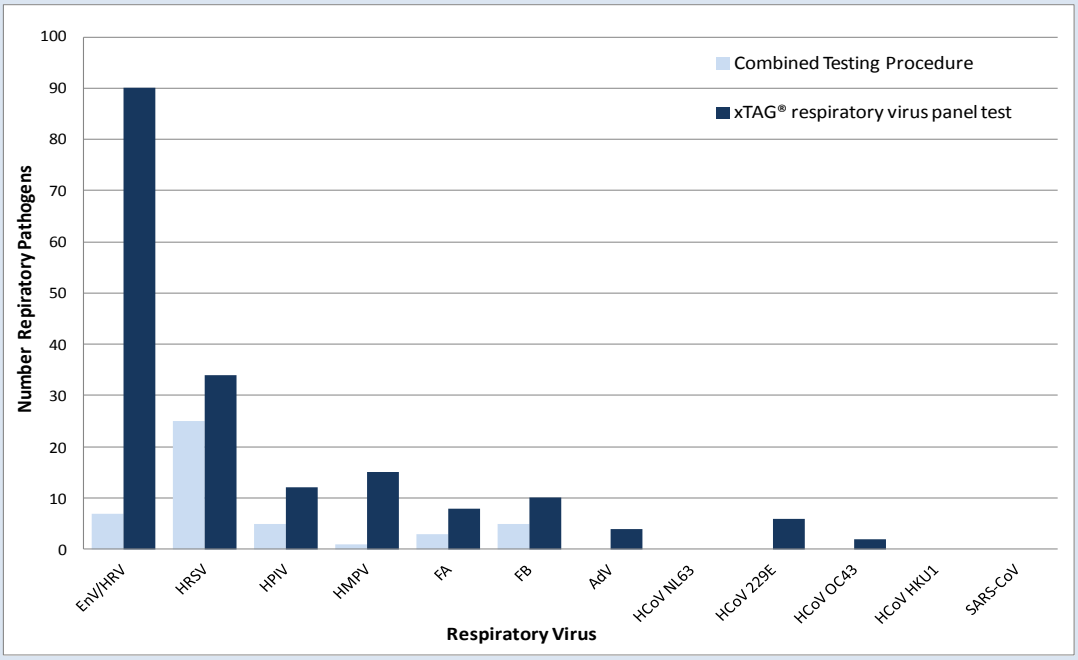


Figure 5.2. Prevalence of respiratory viruses detected by the xTAG® respiratory virus panel test and the combined testing procedure of virus culture, direct immunofluorescence, and NOW® RSV Test.

Overall, 136 additional respiratory viruses were detected in 114/201 (57%) specimens by the xTAG® RVP test (Figure 5.2). The respiratory viruses detected by the xTAG® RVP test were identified as a primary, secondary or tertiary pathogen based on the strength of the MFI signal (Figure 5.3). The following agents were detected in order of frequency as a primary pathogen (n, % of positives): enterovirus/rhinovirus (n = 73, 40.3%), HRSV subtype A (n = 20, 11.0%), HRSV subtype B (n = 14, 7.7%), HMPV (n = 13, 7.2%), influenza virus type B (n = 10, 5.5%), HPIV type 3 (n = 6, 3.3%), influenza virus type A H1 (n = 5, 2.8%), HPIV type 1, influenza virus type A H3 and HCoV 229E (n = 2, 1.1%), HPIV type 2, HPIV type 4, H0CoV OC43, influenza virus no-specific type A (n = 1, 0.6%) (Figure 5.3).

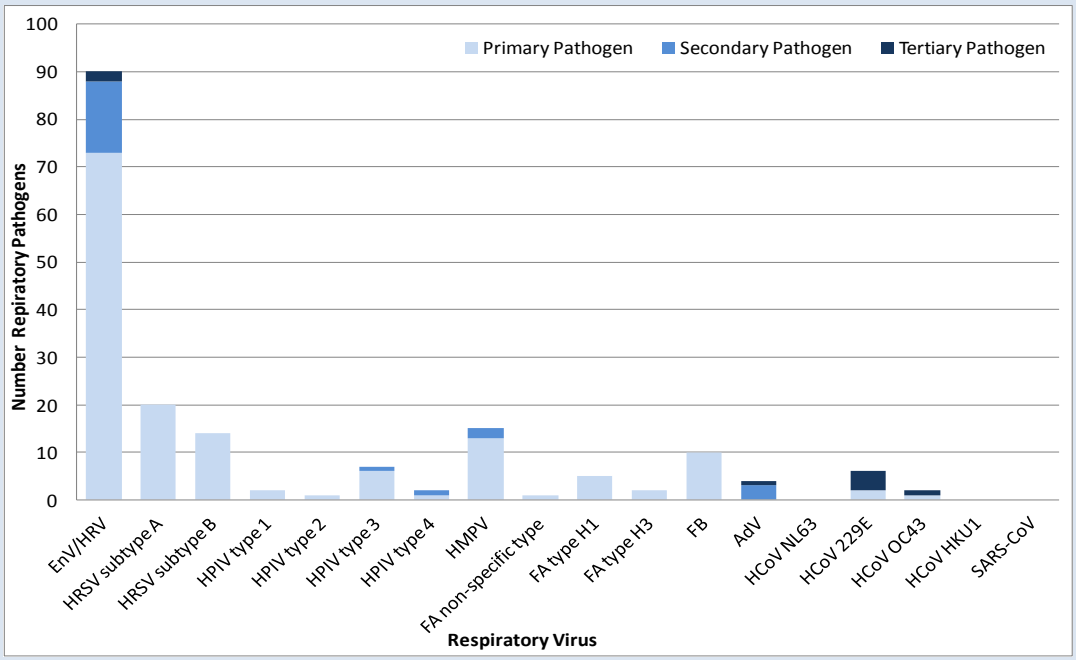


Figure 5.3. Prevalence of respiratory viruses detected by the xTAG® respiratory virus panel test. The columns correspond to the prevalence of individual respiratory viruses detected in nasopharyngeal aspirate samples by the xTAG® respiratory virus panel test. Viruses were identified as a primary, secondary or tertiary pathogens based on the test signal with Median Fluorescent Intensity (MFI). Abbreviations: EnV/HRV, enterovirus/human rhinovirus, HRSV, human respiratory syncytial virus; HPIV, human parainfluenza virus; HMPV, human metapneumovirus; FA, influenza virus type A; FB, influenza virus type B; AdV, adenovirus; HCoV, human coronavirus; SARS-COV, severe acute respiratory syndrome coronavirus.

5.4.3. Comparison of the xTAG® RVP test with an in-house multiplex real-time RT-PCR assay for the diagnosis of respiratory virus infection

Comparison of the xTAG® RVP test and an in-house multiplex RT-PCR for the diagnosis of respiratory virus infection was performed using 201 frozen aliquots of xTAG® RVP tested clinical specimens. The in-house multiplex RT-PCR comprised 3 separate panels for the diagnosis of 12 different respiratory virus types and subtypes including all generic influenza virus A subtypes (H1-H15) and specifically H5 subtypes; influenza virus type B; HPIV types 1 to 4; HRSV; HMPV; adenovirus; HRV, and human enterovirus. In addition to these mutual respiratory virus targets, the xTAG® RVP test incorporated the following viral targets: HCoV OC43, HCoV 229E, HCoV NL63, HCoV HKU1, and SARS-CoV, and offered additional subtyping capabilities for influenza virus type A subtypes H1, H3, and H5, and HRSV subtypes A and B. The xTAG® RVP test used a single set of primers for detection of HRV and enterovirus, and therefore could not discriminate between these viruses in contrast to the in-house multiplex

RT-PCR assay. The results generated by in-house multiplex RT-PCR assay for these targets were combined for comparative analysis with the xTAG® RVP test. Similarly, the results obtained for influenza A subtypes H1, H3, and H5, and HRSV subtypes A and B were combined in order to generate a dataset that was compatible with data produced by the in-house multiplex RT-PCR assay. In total, a respiratory virus was identified in 151/201 (75.1%) respiratory specimens by the xTAG® RVP test compared with 134/201 (66.7%) specimens by in-house multiplex RT-PCR; no respiratory virus was identified in 50/201 (24.8%) specimens by the xTAG® RVP test. Concordance between the xTAG® RVP test and in-house multiplex RT-PCR was achieved for 178/201 (88.6%) respiratory specimens (Table 5.4).

Table 5.4. Distribution of results obtained by the xTAG® respiratory virus panel test and in-house multiplex real-time RT-PCR assays for 201 respiratory specimens

No specimens	xTAG® RVP test	in-house multiplex real-time RT-PCR
131	Positive	Positive
47	Negative	Negative
20	Positive	Negative
3	Negative	Positive

Abbreviation: RVP, respiratory virus panel.

Respiratory viruses common to the xTAG® RVP test and in-house multiplex RT-PCR assay including influenza viruses type A and B, HRSV, HPIV types 1 to 4, HMPV, and adenovirus were identified in 131/201 (65.2%) specimens. The performance of the in-house multiplex RT-PCR assay relative to the xTAG® RVP test for diagnosis of respiratory virus infection is shown in Table 5.5 using the xTAG® RVP test as the gold standard. In total, 181 pathogens were detected in 151 specimens by the xTAG® RVP test with the viral targets HCoV 229E and HCoV OC43 identified as aetiological agents responsible for 8/181 (4.4%) infections. In comparison, the in-house multiplex RT-PCR assay diagnosed 146 mutual viruses in 134 specimens (Table 5.5). Discrepancies between the xTAG® RVP test and in-house multiplex RT-PCR assay were most prominent for HRSV and enterovirus/rhinovirus. Respiratory virus infection attributed to HRSV was diagnosed in an additional 9 respiratory specimens by the xTAG® RVP test while an extra 15 infections with enterovirus/rhinovirus were identified by the xTAG® RVP test in comparison to the in-house multiplex RT-PCR assay. The overall sensitivity and specificity of the in-house multiplex RT-PCR assay in comparison to the xTAG® RVP test as the gold standard was 82.7% and 99.6%, respectively.

Table 5.5. Performance of the xTAG® respiratory virus panel test and in-house multiplex real-time RT-PCR assay for diagnosis of respiratory virus infection in 201 respiratory specimens.
The in-house multiplex real-time RT-PCR assay was compared to the xTAG® respiratory virus panel test as gold standard for viruses common to both tests.

Virus	RT-PCR + xTAG +	RT-PCR – xTAG +	RT-PCR + xTAG –	RT-PCR – xTAG –	Sensitivity (%)	Specificity (%)
EnV/HRV	73	17	1	110	81.1	99.1
HRSV	25	9	1	166	73.5	99.4
HPIV	10	2	0	189	83.3	100.0
HMPV	14	1	0	186	93.3	100.0
FA	8	0	0	193	100.0	100.0
FB	10	0	0	191	100.0	100.0
AdV	1	3	3	194	25.0	98.5

Abbreviations: EnV/HRV, human enterovirus/human rhinovirus, HRSV, human respiratory syncytial virus; HPIV, human parainfluenza virus; HMPV, human metapneumovirus; FA, influenza virus type A; FB, influenza virus type B; AdV, adenovirus; XTAG; xTAG® respiratory virus panel test.

5.4.4. Frequency of multipathogen infections in children with acute respiratory infections

In total, 154 NPA samples were included in this retrospective study to re-examine the interactions among multipathogen infections in hospitalised children with acute respiratory tract infection. However, only 140/154 (88.4%) samples were included for further investigation; comprehensive virological and bacteriological screening was incomplete for the remainder of the stored samples. In total, 112/140 (80%) were positive for a respiratory pathogen; no pathogen was identified in 28/140 (20%) NPA samples.

5.4.4.1. Respiratory Viruses

The resulting data of the xTAG® RVP test were analysed in parallel with results obtained during routine investigations for microbial causes of respiratory tract infection. In total, 92/140 (65.7%) specimens were positive for 1 respiratory virus, while 17/140 (12.1%) were positive for 2 respiratory viruses and 3/140 (2.1%) were positive for 3 respiratory viruses by the xTAG® RVP test. Viral co-infections with HMPV accounted for 3/15 (20%) of all HMPV infections. In contrast, viral co-infections with HRSV and enterovirus/rhinovirus accounted for 11/32 (34.4%) and 18/70 (25.8%) infections attributed to these viruses, respectively. Enterovirus/rhinovirus was detected in 16/20 (80%) specimens with 2 or more respiratory viruses. The prevalence of respiratory viruses detected in NPA samples by the xTAG® RVP test is shown in Figure 5.4. Enterovirus/rhinovirus was detected in 70/140 (50%) specimens

by the xTAG® RVP test followed by HRSV subtype A and B (32/140, 22.9%), HPIV types 1 to 4 (10/140, 7.1%), and HMPV (9/140, 6.4%) (Figure 5.4). Respiratory viruses detected by the xTAG® RVP test were identified as a primary pathogen, secondary or tertiary pathogen based on the strength of the MFI signal. The following agents were detected in order of frequency as a primary pathogen (n, % of positives): enterovirus/rhinovirus (54/140, 38.6%), HRSV type A (19/140; 13.6%), HRSV type B (13/140; 9.3%), HMPV (8/140; 5.7%), HPIV type 3 (6/140; 4.3%), influenza virus type H1 (3/140; 2.1%), influenza virus type B, HCoV 229E, and HPIV type 1 (2/140; 1.4%), HPIV type 4, influenza virus type A H3, HCoV 229E (1/140; 0.7%) (Figure 5.4).

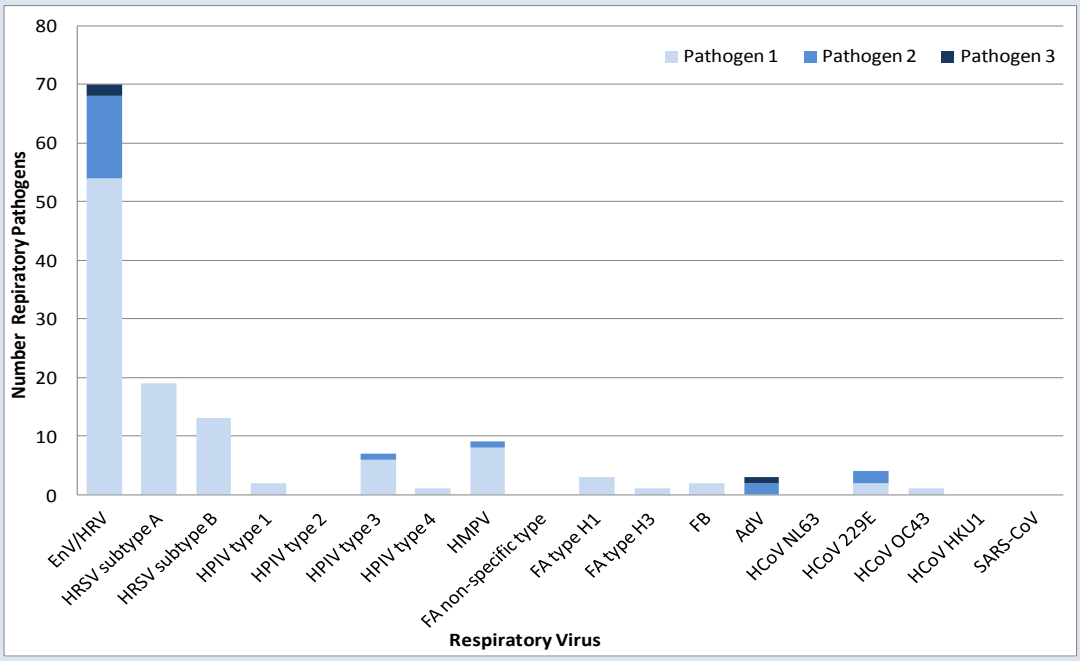


Figure 5.4. Prevalence of respiratory viruses detected by the xTAG® respiratory virus panel in children with acute respiratory tract infection.

The columns correspond to the prevalence of individual respiratory viruses detected in nasopharyngeal aspirate samples by the xTAG® respiratory virus panel test. Viruses were identified as a primary pathogen, secondary or tertiary pathogen based on the test signal with Median Fluorescent Intensity (MFI). Abbreviations: EnV/HRV, enterovirus/human rhinovirus; HRSV, human respiratory syncytial virus; HPIV, human parainfluenza virus; HMPV, human metapneumovirus; FA, influenza virus type A; FB, influenza virus type B; AdV, adenovirus; HCoV, human coronavirus; SARS-COV, Severe acute respiratory syndrome coronavirus.

5.4.4.2. Respiratory Bacteria

Respiratory bacteria were detected in 102/140 (72.9%) NPA samples by bacterial culture. In total, 78/140 (55.7%) samples were positive for 1 bacterial pathogen while 21/140 (15.0%) were positive for 2 bacterial pathogens; mixed infections with 3 bacterial pathogens were uncommon (3/140; 2.1%). No significant bacterial pathogen was isolated from 36/140 (25.7%) NPA samples and no bacterial pathogen was isolated from the remaining 2/140 (1.4%) samples. *H. influenzae* was the most common single bacterial pathogen isolated from NPA samples (41/140; 29.3%) followed by *S. pneumoniae* (38/140; 27.1%); *S. aureus* (19/140; 13.6%), and *M. catarrhalis* (18/140; 12.9%). *H. influenzae* and *S. pneumoniae* were the dominant combination with in NPA samples with mixed bacterial aetiology (17/140; 12.1%).

5.4.4.3. Bacterial and Viral Co-infections

Bacterial and viral co-infections (87/140; 62.1%) were more numerous than viral mono- and co-infections (25/140; 17.9%) and bacterial mono- and co-infections (15/140; 10.7%). Co-infections with a single viral and bacterial co-pathogen (54/140; 38.6%) were more common than infections with multiple viral and bacterial pathogens (33/140; 23.6%) (Figure 5.5). The associations among respiratory viruses and bacteria in children attending the Norfolk and Norwich University Hospital with ARTI are shown in Appendix IV.

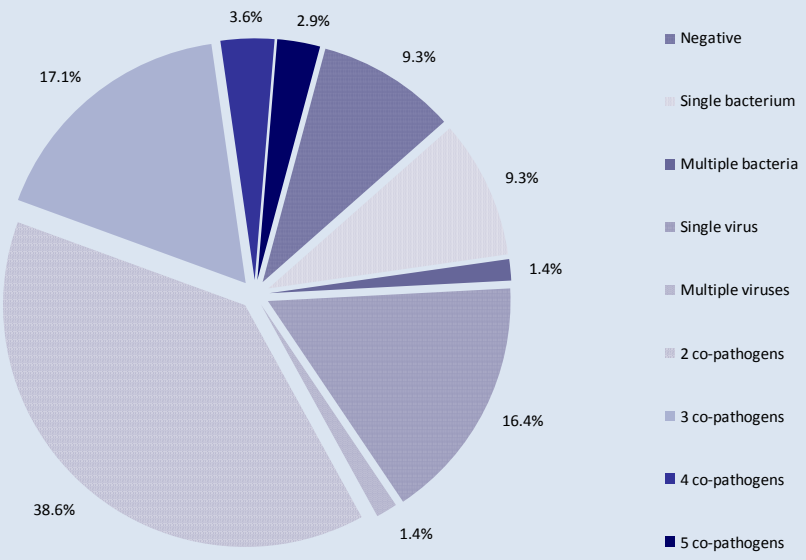


Figure 5.5. Aetiologial agents identified by the xTAG® respiratory virus panel and bacterial culture in nasopharyngeal aspirate samples collected from children ≤18 years attending the Norfolk and Norwich University Hospital with symptoms of acute respiratory tract infection between between October 2007 and December 2008.

Significant associations between pathogen pairs were determined using Spearman's Rank Correlation Test. Significant associations were demonstrated for six pathogen pairs (Table 5.6). The presence of *H. influenzae* was positively correlated with the presence of HMPV (Spearman correlation coefficient = 0.343; p = 3.29E-05) and *S. pneumoniae* (Spearman correlation coefficient = 0.207; p = 1.40E-02). Positive correlations were also demonstrated between HCoV and AdV (Spearman correlation coefficient = 0.237; p = 4.75E-03) and enterovirus/rhinovirus and *S. pneumoniae* (Spearman correlation coefficient = 0.225; p = 7.56E-03). Negative correlations were observed between enterovirus/rhinovirus and HRSV (Spearman correlation coefficient = -0.204; p = 1.56E-02) and influenza virus type A, (Spearman correlation coefficient = -0.171; p = 4.28E-02), respectively (Table 5.6).

Table 5.6. Associations between aetiologial agents identified by the xTAG® respiratory virus panel and bacterial culture in nasopharyngeal aspirate samples collected from children ≤18 years attending the Norfolk and Norwich University Hospital with symptoms of acute respiratory tract infection between October 2007 and December 2008. Associations were determined by Spearman's rank correlation coefficient.

	HMPV	HRSV	HPIV	FA	FB	HCoV	ADV	RV/EV	SPNE	HINF
HMPV	1.00	-0.143	-0.073	-0.045	-0.032	-0.050	-0.039	-0.149	0.036	0.343**
HRSV		1.00	-0.151	-0.093	-0.066	0.079	0.037	-0.204*	0.165	-0.014
HPIV			1.00	-0.048	-0.033	0.096	-0.041	-0.111	0.018	0.126
FA				1.00	-0.021	-0.033	-0.025	-0.171*	-0.105	0.078
FB					1.00	-0.023	-0.018	0.000	-0.073	-0.077
HCoV						1.00	0.237**	0.038	-0.031	0.045
AdV							1.00	0.049	0.132	0.122
RV/EV								1.00	0.225**	0.110
SPNE									1.00	0.207*
HINF										1.00

Probabilities of association by chance (p-values) are shown with figures indicating significant correlations at the 0.01 (**) and 0.05 (*) level highlighted in red. Abbreviations: HMPV, human metapneumovirus; HRSV, human respiratory syncytial virus; HPIV, human parainfluenza virus; FA, influenza virus type A; FB, influenza virus type B; HCoV, human coronavirus; AdV, adenovirus; RV/EV, human enterovirus/human rhinovirus; SPNE, *Streptococcus pneumoniae*; HINF, *Haemophilus influenzae*.

5.5. Discussion

Many of the advances in clinical virology have come about because of the ability to grow viruses in the laboratory (Jeffery and Aarons, 2009). With the appropriate specimens and optimal cell lines, this technique can be highly sensitive and specific, with a presumptive diagnosis made based on a characteristic cytopathic effect and the particular diagnosis confirmed by haemadsorption or by immunofluorescence using a virus-specific antibody labelled with a fluorescent dye (Jeffery and Aarons, 2009). The judicious selection of two or three cell lines will allow the detection of the majority of cultivable viruses of clinical importance (Jeffery and Aarons, 2009). While virus isolation has always been recognised as a relatively late diagnosis, involving a few days to weeks in culture, it is no longer acceptable, particularly when the goal of the modern clinical virology laboratory is the provision of a rapid and comprehensive diagnostic service to facilitate the implementation of prompt clinical action (Ogilvie, 2001). Furthermore, the unreliability of culture for the isolation of slow growing or labile viruses as well as several of the newer respiratory viruses, which fail to proliferate in traditional virus cultures as a result of fastidious growth requirements, has brought the diagnostic value of virus culture under further criticism (van den Hoogen *et al*., 2001; Leland and Ginocchio, 2007; Mahony, 2008; Pavia, 2011; Storch, 2000; Talbot and Falsey, 2010). Indeed, the problems that contributed to the anonymity of HMPV, namely poor replication in continuous cell lines, very slow replication kinetics *in vitro* in contrast to other human respiratory viruses, trypsin dependence, and absence of haemagglutinating activity with turkey, chicken, or guinea pig erythrocytes, largely pertain to diagnostic virology laboratories (van den Hoogen *et al*., 2001).

The development of molecular methods has revolutionised the diagnosis of respiratory virus infections. It is widely reported that utilisation of molecular methodology for the diagnosis of respiratory virus infections offers greater sensitivity with a resultant increase in the detection rate of respiratory viral pathogens in comparison to traditional diagnostic methods (Balada-Llasat *et al.,* 2010; Coiras *et al*., 2004; Gadsby *et al*., 2010; Kehl *et al*., 2001; Kuypers *et al*., 2006; 2009; Marshall *et al*., 2007; Nolte *et al*., 2007; Templeton *et al*., 2004; Weinberg *et al*., 2004) and the findings in the present study provide no exception. Excepting HRSV, detection rates among HPIV types 1 to 4, influenza virus type A and influenza virus type B, which represent the principal targets of traditional diagnostic methods, achieved using a combination of virus culture and DFA were much lower than those obtained by the xTAG® RVP test and in-house multiplex real-time RT-PCR assay. Historically, HRSV has been more difficult to culture than other respiratory viruses since it is quickly inactivated in samples that

are not kept refrigerated or inoculated into virus culture within a short time after specimen collection (Leland and Ginocchio, 2007; Mahony, 2008). This was particularly problematic for the Microbiology Department as an off-site facility where specimen transport was required and could provide an explanation for the low detection rate of HRSV achieved. In contrast, virus culture is most useful for relatively hardy viruses, such as influenza virus, which can survive transportation to a laboratory (Talbot and Falsey, 2010). The microscopic examination of the unstained cell culture monolayer for CPE is the simplest and most widely used criterion for infection in virus culture (Condit, 2007; Leland and Ginocchio, 2007). The CPE may be sufficiently distinctive to allow unequivocal identification of a virus but some viruses including influenza and parainfluenza viruses may produce only subtle morphologic changes in the cell monolayer and so in these cases other methods must suffice (Ashley, 1999; Condit, 2007; Storch, 2007; Winn *et al*. 2006). Detection of these viruses was performed by haemadsorption, which utilises the erythrocyte binding capabilities of the viral attachment glycoprotein, haemagglutinin (Storch, 2007). However, both techniques require considerable technical expertise, which at times was in shortage. Further difficulties with diagnosis of influenza virus and parainfluenza virus infections related to the change in cell line in routine diagnostic use. The PLC/PRF/5 cell line was introduced as an alternative to primary rhesus monkey kidney (RhMK) cells following cessation of the use of this cell line for ethical reasons in 2006 (HPA 2010a). This cell line was received infrequently as a monolayer with resultant rapid cell degeneration, which presented difficulties in the interpretation of the haemadsorption test. This combination of factors may provide an explanation for the reduction in detection rate of influenza and parainfluenza viruses in relation to the xTAG® RVP test. Nevertheless, diagnosis of virus respiratory infection was supported by non-culture methods including detection of viral antigens by DFA and the BinaxNOW® RSV test. DFA tests for the detection of HRSV surpass the diagnostic sensitivity of conventional virus culture for the diagnosis of HRSV infection (Leland and Ginocchio, 2007; Mahony, 2008). Indeed, the majority of HRSV infections were diagnosed by DFA in combination with the BinaxNOW® RSV test, which has comparable sensitivity to DFA for the detection of HRSV (Jonathan, 2006; Mackie *et al*., 2004). However, another plausible explanation for the high false-negative rate achieved for HRSV pertains to BinaxNOW® RSV test. This test was performed as a rapid point of care screening test within the acute hospital setting as a useful adjunct to the diagnosis of HRSV infections in symptomatic neonatal and paediatric patients under the age of five years. Although the need for technical expertise is advertised as being minimal for performance of this rapid test, testing that is carried out by technicians or other personnel who are less experienced with test kits, especially in reading results that are weakly positive, yields lower

sensitivity and specificity relative to cell culture (Leland and Ginocchio, 2007). This is a common finding amongst institutions that utilise point-of-care testing (POCT) (Mackie *et al*., 2004) including our own and underlines the necessity for rigorous training and quality control for even the most user-friendly assay (Mackie *et al*., 2004). Furthermore, it is important these tests be performed in conjunction with routine testing in order to ameliorate the implications of a POCT false negative result and allow diagnosis of mixed viral respiratory infections (Khanom *et al*., 2011).

The reported sensitivities of IF testing for the other respiratory viruses compared to virus isolation in cell culture are lower than those reported for HRSV, and vary considerably from report to report, which reflects the variable performance of the test in relation to virus type, specimen quality, and interpretation of a positive result, which is subjective and requires a great deal of technical skill (Leland and Ginocchio, 2007; Syrmis *et al*., 2004). However, it is also important to consider that older adults generally have lower viral loads in their respiratory secretions, which may affect the sensitivity of traditional diagnostic methods (Talbot and Falsey, 2009). As a result, the recognition and diagnosis of infection due to many viral respiratory pathogens in older adults can be elusive (Talbot and Falsey, 2009). Molecular diagnostic techniques now offer the potential to diagnose a greater breadth of viral respiratory infections including viruses not routinely detected by traditional diagnostic methods, such as coronavirus and HMPV, as well as provide a more accurate diagnosis in this specific population (She *et al*., 2010; Talbot and Falsey, 2009). This is especially useful in elderly patients for whom viruses can cause severe to fatal respiratory disease (Boivin *et al*., 2007; Liao *et al*., 2011; She *et al*., 2010) with the additional benefit within the nursing home setting to identify sources of outbreaks (Talbot and Falsey, 2009).

The failings of traditional diagnostic methods for the diagnosis of respiratory virus infections are perhaps most pertinent to HRV and HCoV. Historically, HRV culture was fraught with unreliability (Arden and MacKay, 2010). The range of susceptibility of different cell lines and even different lots of the same cells made HRV isolation difficult as more than one cell line was often required for optimal sensitivity. Furthermore, HRV serotypes lack a common group antigen, making the possibility of broadly reacting antibodies unlikely. With the advent of diagnostic molecular methods, it became apparent that HRV infections are the most frequent of viral respiratory infections, even in hospitalised children (Arden and Mackay, 2010). Comparison with the xTAG® RVP test and in-house multiplex real-time RT-PCR assay in the present study exemplifies the failings of virus culture for the diagnosis of

HRV infection, which was a severely under-represented aetiologic agent by this method. In contrast, picornaviruses were detected in 44.7% and 36.3% of specimens by the xTAG® RVP and multiplex RT-PCR assay, respectively. A breakdown of the results generated by the in-house multiplex RT-PCR assay for the specific virus targets revealed that human enterovirus and HRV were detected in 8.0% and 29% of specimens, respectively. This dramatic increase in the frequency of HRV detection emphasises the improved sensitivity that molecular methods offer comparative to cultivations methods and the clinical importance of this virus as a respiratory pathogen (Mackay, 2008).

The xTAG® RVP test and in-house multiplex real-time RT-PCR assay significantly improved the detection of HMPV in comparison to DFA, which was performed using the IMAGEN™ hMPV immunofluorescence test (Oxoid Ltd, Hampshire, UK). The xTAG® RVP test and in-house multiplex real-time RT-PCR assay enabled the positive diagnosis of HMPV infection in an additional 7% and 6.5% of specimens, respectively, which were negative by DFA.

The overall sensitivity and specificity of the in-house multiplex RT-PCR assay in comparison to the xTAG® RVP test as the gold standard was 82.7% and 99.6%, respectively, reflecting a range of sensitivities among the common viral targets. Discordant results between these tests were most prominent for the picornaviruses and HRSV, which perhaps represents suboptimal primer binding or the difficulty in accommodating strain diversity with a single probe and RNA viruses, which exhibit considerable genetic diversity, present particular challenge (Ratcliff *et al*., 2007). It is difficult to comment on the results obtained for adenovirus by both the xTAG® RVP and multiplex RT-PCR assay due to the limited number of specimens that were positive for this virus target and in the absence of re-testing discordant results. However, the inadequacy of the xTAG® RVP in the detection of adenovirus is reported previously (Gadsby *et al*., 2010; Pabbaraju *et al*., 2008). Pabbaraju *et al*., (2008) observed that there was a significant reduction in the detection of adenovirus in younger patients by the xTAG® RVP; however, there was no significant difference based on sample type, and analysis of serotypes was too limited to draw complete conclusions. Suboptimal primer binding in particular adenovirus serotypes may also account for the low sensitivity for observed for this virus target (Gadsby *et al*., 2010).

The results of the xTAG® RVP test and in-house real-time RT-PCR assay clearly indicate why the advent of molecular methods have changed our view of the aetiology and clinical spectrum of viral respiratory infections (Olofsson *et al*., 2011). Molecular methods have revolutionised the diagnosis of viral respiratory infections not only because of the new

unparalleled detection sensitivities achieved, but also because of the option of simultaneous assay for a great number of infectious agents, at a reasonable cost (Olofsson *et al*., 2011). Molecular methods have revealed that multiple respiratory virus infections are more frequent than previously expected (Olofsson *et al*., 2011) and within the present study the presence of multiple infectious agents was the norm, not the exception, among acute phase respiratory specimens tested in concordance with earlier investigations of the true pathogen spectrum of respiratory infections (Brunstein *et al*., 2008). Respiratory infections with 2 co-pathogens were diagnosed in 38.6% of specimens and accounted for the largest proportion of co-infections. However, overall co-infections with 2 or more infectious agents accounted for 65% of all respiratory infections with the remaining 25.7% of infections attributed to a single respiratory pathogen.

One of the most exciting interactions to emerge from the application of molecular methods to the diagnosis of respiratory viruses pertains to HRV. These viruses, previously called coryzaviruses, ECHO 28-rhinovirus-coryzaviruses (ERCs), muriviruses, enterovirus-like viruses, nasal secretion agents and Salisbury strains are the most common cause of ARTI and URTI, traditionally defined as 'common colds' (Mackay, 2008). However, recent wider use of molecular detection methods in viral diagnostic screening has contributed to the reappraisal of HRV, as has the quite unexpected discovery of an entirely novel species of HRV designated species C, which is refractory to previously used virus isolation methods (Simmons *et al*., 2010; Wisdom *et al*., 2009). Ironically, this forgotten diagnostic target is now the focus of investigations in which other viruses are sometimes forgotten (Arden and Mackay, 2010). Historically, the elevated involvement of HRV in co-infections with other viruses in acute respiratory infections provided a reason to diminish the causal role of HRV infections in clinical outcomes other than the common cold (Greer *et al*., 2009). However, an increasing weight of evidence disputes these superficial observations and indirectly indicates an aetiological role for HRV in respiratory disease (Greer *et al*., 2009). The most striking observation amongst investigations conducted so far is the predominance of co-infections with HRSV despite differences in the study years and molecular methodology utilised, which has provoked a rethink of the role of these viruses as unobtrusive passengers in favour of these viruses driving respiratory illness (Arden and Mackay, 2010). Evidence for contemporaneous detections with other respiratory viruses including adenovirus, HCoV, HBoV, HMPV, influenza virus type A, HPIV, and the polyomaviruses KIPyV and WUPyV are reported (Brunstein *et al*., 2008; Greer *et al*., 2009; Wisdom *et al*., 2009), which reveal a consistent pattern of HRV detection associated with a reduced likelihood of co-detection

with these viruses (Greer *et al*., 2009). No correlation was observed between HMPV and HRV within the present study although negative correlations were observed between enterovirus/rhinovirus and HRSV (Spearman correlation coefficient = -0.204; p = 1.56E-02) and influenza virus type A, (Spearman correlation coefficient = -0.171; p = 4.28E-02), respectively. This has prompted a new hypothesis, which contrary to the role that has historically held viruses as instigators of infection suggests that HRV infection may offer protection as a paradoxical viral commensal and temporarily protect its host from infection by other, often more cytopathic, viruses (Greer *et al*., 2009). Indeed, the unexpected delay in the emergence and spread of the novel pandemic (H1N1) 2009 influenza virus in several European countries is attributed to interference with other viruses. HRV is suggested as the most probable culprit; a hypothesis supported by a significant inverse relationship between HRV and the 2009 H1N1 virus irrespective of the time period and the age group analysed (Ånestad and Nordbø, 2009; 2011; Casalegno *et al*., 2010; Linde *et al*., 2009). Few co-infections with HMPV and other respiratory viruses were observed within the present study, which may explain the association absence between HMPV and HRV. Nevertheless, these viruses represented the most common pairing within the present study. Equally, the finding that HMPV is often associated with a reduced likelihood co-detection of other respiratory viruses (Greer et al., 2009) may support the limited number of co-infections observed.

Some have ascribed the increased frequency of viral co-detection to simple coincidental overlap of epidemic seasons (Greer *et al*., 2009). However, analysis indicates that these associations do not occur by chance, which suggests more complex mechanisms are responsible (Greer *et al*., 2009). HRV is considered to mediate illness through immunological rather than cytopathic mechanisms (Greer *et al*., 2009). HRV replication, as for HRSV and influenza viruses, produces double-stranded RNA (dsRNA) replicative intermediates; molecules that mediate triggering of IFN-stimulated genes inducing an antiviral state (Greer *et al*., 2009). It is hypothesised that the putative interference patterns observed are related to the induction of this innate IFN response by HRV infection in the respiratory tract, which creates a hostile environment and shields neighbouring cells from infection with other viruses (Greer *et al*., 2009; Wisdom *et al*., 2009). Even though HRSV, through expression of NS-1, can prevent IFN induction on infection of a cell, this countermeasure may be largely ineffective in a respiratory tract already induced into an antiviral state by prior infection with HRV (Wisdom *et al*., 2009). If, however, HRSV infected the respiratory tract first, then this would have no effect on the subsequent susceptibility of the individual to HRV (Wisdom *et al*., 2009). The rapid and highly cytopathic replication cycle of rhinoviruses and enteroviruses

that seems designed to infect and escape from cells before IFN-mediated responses become effective, may indeed have a more general interfering effect on more sensitive RNA and DNA viruses that infect the respiratory tract (Wisdom *et al*., 2009). Like HRSV, successful colonisation of the respiratory tract by coronaviruses, influenza viruses, and parainfluenza viruses is dependent on a wide variety of evolved mechanisms to evade intracellular defences that are ineffective in presensitised cells induced by IFN into an antiviral state (Wisdom *et al*., 2009).

Almost all mammalian viruses have developed strategies to avoid host immune responses by attacking the IFN system (Ren *et al*., 2011). HRSV preferentially inhibits IFN-α/β signalling by expression of viral non-structural proteins NS1 and NS2. Thus, HRSV infection causes a marked decrease in STAT2 levels and the consequent downstream IFN-α/β response (Lo *et al*., 2005). In contrast, HMPV lacks the non-structural proteins NS1 and NS2, which suggests that the virus may encode unique mechanisms to subvert viral clearance (Dinwiddie and Harrod, 2008) and in fact, HMPV infection interferes with type I IFN signalling, leading to inhibition of IFN-**β** signalling transduction (Ren *et al*., 2011). The inhibitory effect of HMPV on type I IFN signalling occurs at different levels of the signalling cascade, as the virus partially blocks JAK1 and TYK2 gene transcription, facilitates JAK1 and TYK2 degradation, and lowers IFNAR1 membrane expression, ultimately leading to inhibition of STAT1 and STAT2 activation (Ren *et al*., 2011). However, the differences in the interplay between HMPV and host immune defences in comparison to HRSV does not seem to offer any additional advantage to HMPV in evading intracellular defences in presensitised cells induced by IFN into an antiviral state by prior HRV infection (Wisdom *et al*., 2009).

The effect of the order in which infections are acquired may additionally influence the outcomes of co-infections with adenoviruses. These viruses express a plethora of evasion molecules that substantially influence the intracellular environment of the cell that they infect as well as a broader paracrine effect on cytokine production in the respiratory tract and induction of local immunity (Wisdom *et al*., 2009). The frequent long-term persistence of adenovirus infections suggest that a more permissive environment for infection and replication by other viruses may exist in the respiratory tract of adenovirus-infected individuals, and underlie the increased detection frequencies of HRSV, HRV and other respiratory viruses in coinfected subjects (Wisdom *et al*., 2009). This infectivity enhancing mechanism may offer a pertinent explanation for the positive correlation observed between HCoV and adenovirus (Spearman correlation coefficient = 0.237; p = 4.75E-03) within the

present study, which represents a little acknowledged association at present. However, examination of the literature suggests co-infections with adenovirus deserve heightened recognition. Numerous studies have reported a high rate of viral co-infection with adenovirus (Choi *et al*., 2006; Coyle *et al*., 2004; Jennings *et al*., 2004; Martin *et al*., 2012; Wong *et al.,* 2008) but this finding is not universal (Cilla *et al*., 2008). Furthermore, severe respiratory infections following co-infection by HRSV and adenovirus are documented, which suggests that adenovirus has the potential to promote a more severe evolution of infection since long-term sequelae associated with HRSV infection in previously healthy infants and children are rarely reported (Hirschheimer *et al*., 2002; Kaida *et al*., 2007; Massie and Armstrong, 1999; Murtagh *et al*., 2009). In contrast, co-infections between HMPV and adenovirus are not associated with more severe infection (Kaida *et al.*, 2007). Interestingly, Martin *et al*., (2012) found adenovirus was most commonly detected in co-infections followed by HCoV but illnesses with multiple virus detections were correlated with less severe disease (Martin *et al*., 2012). Moreover, adenovirus viral quantities were significantly reduced in samples from multiple virus illnesses compared with single virus illnesses (Martin *et al*., 2012). Perhaps the relationship between HRV and adenoviruses epitomises the relationship between adenovirus and other respiratory viruses. Evidence from studies amongst military recruits suggests a strong negative association exists between HRV and adenovirus (Wang *et al*., 2010). In recruits, high rates of HRV within healthy recruits was associated with low rates of adenovirus and conversely adenovirus febrile respiratory illness was associated with decreased HRV titres. The inference from these findings is the existence of some form of interference between these viruses; a refractory period during HRV infections and shedding may thwart subsequent adenovirus infection while the fevers induced by adenovirus infection may be sufficient to restrict replication of HRV (Wang *et al*., 2010). In line with this theory, Martin *et al*., (2012) found that HRSV, influenza virus type A HPIV type 3, and HMPV viral loads were consistently high whether or not another virus was detected. These associations may offer insight into which virus predominates in a multiple virus illness. Notably, 82% of co-infections consisted of one virus from the group with consistently high viral load combined with an alternate virus (HCoV, HPIV type 1, or adenovirus). This may suggest a possible model for virus co-infections that include one predominant virus and one virus that is present at a lower quantity and does not confer increased severity (Martin *et al*., 2012). Unfortunately, the total number of viral co-infections was likely underestimated, as the presence of HRV was not included in the evaluation but the relationship between viral load and multiple virus infections was virus specific within the limits of the study (Martin *et al*., 2012).

While *S. pneumoniae* and HRV are among the most common aetiologic agents in children with community-acquired pneumonia (Juvén *et al*., 2000; Nascimento-Carvalho *et al*., 2008) few studies have addressed the pathogenesis of mixed rhinoviral-bacterial infections (Peltola *et al*., 2008), which is perhaps attributed to the legacy of neglect that has surrounded HRV. Nonetheless, it seems that the use of molecular detection methods has inspired a reappraisal of HRV in viral-bacterial co-infections as it has viral co-infections. Recent albeit limited studies, report that HRV and *S. pneumoniae* were the most commonly found combination of virus and bacterial pathogen in children with community-acquired pneumonia (Honkinen *et al*., 2011; Lahti *et al*., 2009). Another recent study found a temporal association between HRV circulation in the community and IPD in children younger than 5 years of age, suggesting that HRV infection may be a contributor in the development of IPD in this population (Peltola *et al*., 2011). While mixed HRV and *S. pneumoniae* infection was associated with severe pneumonia in adults with community-acquired pneumonia (Jennings *et al*., 2008]. These studies suggest an association exists between these respiratory pathogens and indeed within the present study a positive correlation was observed between enterovirus/rhinovirus and *S. pneumoniae* (Spearman correlation coefficient = 0.225; p = 7.56E-03). *S. pneumoniae* adheres to the airway epithelial cells and vascular endothelial cells via binding to a receptor for the G protein–coupled platelet-activating factor (PAF), *N*-acetylgalactosamine b-1–4-galactose, or *N*-acetylgalactosamine b-1–3-galactose (Ishizuka *et al*., 2003). Activation of human epithelial and endothelial cells by IL-1α and TNF–α produced in inflamed sites induces the adherence of this bacteria to PAF receptor (PAF-R) (Ishizuka *et al*., 2003). Studies demonstrate that HRV infection can reach, penetrate, and replicate in lower airway epithelium and induce the production of a variety of inflammatory cytokines by the alveolar and bronchial epithelial cells as well as surface expression PAF-R, which increases the adherence of *S. pneumoniae* to human tracheal epithelial cells (Ishizuka *et al*., 2003; Papadopoulos *et al*., 2000). Furthermore, impairment of cytokine responses to bacterial lipopolysaccharide and lipoteichoic acid by alveolar macrophages in response to infectious HRV represents a novel mechanism by which HRV can impair the innate immune response in alveolar macrophages and thereby provide an environment that facilitates additional bacterial infection. Other mechanisms likely also play a role in the development of secondary bacterial pneumonia during or after HRV infection (Peltola *et al*., 2008) as it is clear that new battles are underway against these most abundant and equally misunderstood viral foes (Mackay, 2008). These finding also lend interesting possibilities to the role of bacteria in multiple virus infections, which are severely under-represented in studies conducted to date. It is plausible that the relationship bacteria and viruses in

multiple virus infections may provide the missing link in the development of severe infections with multiple viruses including HMPV.

CHAPTER SIX

6. Molecular epidemiology of human metapneumovirus

6.1. Introduction

The existence of two distinct genetic lineages of HMPV, A and B, circulating within the human population was demonstrated early in the discovery of this previously unknown virus (Boivin *et al*., 2002; Boivin *et al*., 2004; Madhi *et al*., 2003; Peiris *et al*., 2003; Peret *et al*., 2002; Stockton *et al*., 2002; van den Hoogen *et al*., 2001). Subsequent analysis of the nucleotide and predicted amino acid sequences of the F and G protein genes has further addressed the genetic heterogeneity of HMPV and revealed that the two main genetic lineages represent two serotypes of HMPV with further division of each serotype into two genetic sublineages, tentatively named A1, A2, B1 and B2 (van den Hoogen *et al*., 2004b). More recently, evidence has revealed a further bipartition of sublineage A2 into two new genetic clusters, designated A2a and A2b (Huck *et al*., 2006). The F protein is highly conserved exhibiting high percentage identities between members of the same sublineage (nt: 97%–100%, amino acids [aa]: 99%–100%), members of the two different sublineages within each main lineage (nt: 94%–96%, aa: 97%–99%), and between members of the two different main lineages A and B (nt: 84%–86%, aa: 94%–97%) (van den Hoogen *et al*., 2004b). Similarly, the G protein demonstrates a relatively high percentage identity between members of the same sublineage (nt: 93%–100%, aa: 75%–99.5%) and between members of the two different sublineages within each main lineage (nt: 76%–83%, aa: 60%–75%) but is highly variable between members of the two different main lineages A and B (nt: 50%–57%, aa: 30%–37%) (van den Hoogen *et al*., 2004b), which suggests that the G protein is continuously evolving (Bastien *et al*., 2004).

The epidemiology of HMPV is complex and dynamic (Kahn, 2006). Annual circulation of HMPV genotypes, A and B, is observed worldwide and concurrent annual circulation of all four genetic lineages of the virus is common (Huck *et al*., 2006; Ludewick *et al*., 2005; Mackay *et al*., 2006). However, a highly localised and community based phenomenon also exists (Arnott *et al*., 2011b; Gaunt *et al*., 2011; Kahn, 2006) with fluctuating circulation frequencies of genotypes giving rise to frequently observed switching of the predominant circulating genotype over consecutive epidemic seasons (Aberle *et al*., 2010; Gaunt *et al*., 2011). The dynamic nature of localised HMPV epidemics is increasingly highlighted by studies throughout the world (Aberle *et al*., 2010; Agrawal *et al*., 2011; Arnott *et al*., 2011b; Carr *et al*., 2008; Chung *et al*., 2008; Escobar *et al*., 2009; Galiano *et al*., 2006; Legrand *et al*., 2011; Ljubin-Sternak *et al*., 2008; Ludewick *et al*., 2005; Mackay *et al*., 2006; Oliveira *et al*.,

2009; Pitoiset *et al*., 2010; Wang *et al*., 2008). It has become clear that sublineages of HMPV do not persist and that old lineages may be replaced with newly emerging variants (Schildgen *et al*., 2011).

The F protein is a major antigenic determinant that mediates extensive cross-lineage neutralisation and protection (Skiadopoulos *et al*., 2004). Cross-challenge experiments aimed at inducing antibodies against the conserved HMPV F protein in animal models revealed that primary infection with either of the two main genetic lineages of HMPV induced protective immunity against subsequent challenge with homologous or heterologous virus (MacPhail *et al*., 2004; Skiadopoulos *et al*., 2004; van den Hoogen *et al*., 2007). Conversely the glycoproteins G and SH play minor or insignificant roles in stimulating neutralisation and protection (Biacchesi *et al*., 2005) and it is postulated that antigenic variability of the G gene may play an important role in the ability of this virus to escape the pre-existing immune response (Kahn, 2006).

6.2. Aims

In this chapter, the identification of circulating HMPV genotypes and associated sub-genotypes within the locality of Norwich was sought through nucleotide analysis of the highly conserved F gene and the diverse G gene regions of the HMPV genome in order to determine the geographical and temporal distribution of HMPV within this undescribed region. Finally, nucleotide sequence data generated within the present study was compared against data available from the genetic sequence database, GenBank®, to facilitate the assessment of current knowledge on the molecular epidemiology of this complex and dynamic virus.

6.3. Materials and Methods

6.3.1. Overview of study design

The molecular epidemiology and genetic diversity of HMPV within children ≤18 years attending the Norfolk and Norwich University Hospital with symptoms of acute respiratory tract infection between 31st October 2005 and 31st December 2008 was characterised using positive frozen, archived NPA samples that were identified as described previously (Section 4.4.3). In total, 36 positive NPA samples were available for sequence analysis; two samples were excluded from the analysis as insufficient clinical material remained.

6.3.2. Selection of primer sequences for phylogenetic analysis

Primer pairs selected from the F gene ORF and G gene ORF (Table 6.1) corresponding to those published by Huck *et al*., (2006) and Ludewick *et al*., (2005), respectively were selected for phylogenetic analysis. The F gene ORF and G gene ORF forward and reverse primers targeted a 507 bp and 897 bp region within the F gene and G gene of the virus, respectively. The regions of the genome covered by the forward and reverse primers targeting the F gene ORF and G gene ORF were nucleotide positions 3,624–4,130 and 6285 to 7181, respectively.

Table 6.1. Composition of primer pairs selected for phylogenetic analysis.

Primer or probe	Sequence (5'-3')	Target Gene
Forward	GTY AGC TTC AGT CAA TTC AAC AGA AG	Fusion
Reverse	CCT GTG CTG ACT TTG CAT GGG	

Primer sequences for the detection of the fusion (F) gene open reading frames (ORF) of human metapneumovirus (HMPV). Residue Y in the third position of the forward primer represents either a cytosine (C) or a thymidine (T) residue.

Primer or probe	Sequence (5'-3')	Target Gene
Forward	GAG AAC ATT CGR RCR ATA GAY ATG	Glycoprotein
Reverse	AGA TAG ACA TTR ACA GTG GAT TCA	

Primer sequences for the detection of the glycoprotein (G) gene open reading frame (ORF) of human metapneumovirus (HMPV). Residue R in the twelfth, thirteenth, and fifteenth position of the forward primer and the twelfth position of the reverse prime represents either an adenine (A) or a guanine (G) residue. Residue Y in the twenty-first position of the forward primer represents either a C or a T residue.

6.3.3. Nucleic Acid Extraction

All samples were subjected to nucleic acid extraction using the COBAS® AmpliPrep TNAI Kit (Roche Diagnostics Ltd, Burgess Hill, UK) on the COBAS® AmpliPrep Instrument (Roche Diagnostics Ltd, Burgess Hill, UK), according to manufacturer's instructions (Section 2.6.4.4).

6.3.4. Agarose Gel Electrophoresis

Gel electrophoresis was performed using E-Gel® pre-cast 2% agarose gels with SYBR Safe™ DNA gel stain (Invitrogen, Paisley, UK). Each gel contained a single row of 12 sample wells. E-Gel® agarose gels were prepared using the one-step loading method according to the manufacturer's instructions. Samples were prepared by adding 3.3 µl of a 1:10 dilution of TrackIt™Cyan/Yellow Loading Buffer (6X) (Invitrogen, Paisley, UK) that was prepared in deionised water to 6.7 µl of PCR product and 10 µl of deionised water to a final volume of 20

µl. The gel was inserted into the E-Gel® PowerBase™ v.4. Next, 20 µl of each prepared DNA sample was loaded alongside 10 µl of E-Gel® Low Range Quantitative DNA Ladder that was diluted with 10 µl of deionised water. The E-Gel® Low Range Quantitative DNA Ladder consists of five linear double-strand DNA fragments (100-2000 bp). Electrophoresis of 10 µl of the ladder resulted in bands containing 100, 40, 20, 10, and 5 ng of DNA, respectively. Empty wells were loaded with 20 µl of deionised water. The gel was run at a constant voltage of 60-70 volts for 30 minutes. At the end of the run, the gel cassette was removed from the power unit. DNA fragments were visualised under UV light using the MiniBIS Pro Gel Documentation System (Berthold Technologies (U.K.) Ltd, Hertfordshire, UK) in combination with two software packages, GelCapture for imaging acquisition and GelQuant for image analysis that were supplied with the MiniBIS Pro Gel Documentation System.

6.3.5. Conventional RT-PCR using the QIAGEN® OneStep RT-PCR Kit

Conventional block-based RT-PCR was performed using the OneStep RT-PCR Kit (QIAGEN Ltd, Crawley, West Sussex, UK) in order to amplify two independent regions of the F and G gene open reading frames of HMPV for sequencing and phylogenetic analysis. The OneStep RT-PCR Kit contained a specially formulated OneStep RT-PCR Enzyme Mix for reverse transcription and PCR using a unique combination of Omniscript and Sensiscript Reverse Transcriptases that have high affinity for RNA templates and HotStar *Taq* DNA Polymerase in addition to 5X OneStep RT-PCR Buffer containing 12.5 mM $MgCl_2$, dNTP Mix containing 10 mM each of dATP, dCTP, dGTP, and, dTTP, and nuclease-free water. A negative control was included in each experiment. One-step RT-PCR was performed in a final reaction volume of 50 µl containing 10 µl of 1x RT-PCR Buffer, 2.0 µl dNTP Mix containing 400 µM of each deoxyribonucleotide (dNTP), 2.0 µl of RT-PCR Enzyme Mix, 1.5 µl each of F gene ORF or G gene ORF forward and reverse primers, and nuclease-free water to a volume of 45 µl (Table 6.2). The working one-step RT-PCR reaction mix was aliquoted into 0.2 ml reaction tubes (Alpha Laboratories, Hampshire, UK). Finally, 5 µl of viral RNA extract was added exclusive of the NTC. Instead, 5 µl of nuclease-free water was added.

Table 6.2. Preparation of working OneStep RT-PCR Kit reaction mix for 1x reaction.

Reaction Component	Volume (µl) per 50 µl reaction	Stock Concentration	Final Concentration
5 X QIAGEN OneStep RT-PCR Buffer*	10.0	5x	1x
dNTP Mixture	2.0	10 nM each	400 µM each
Forward Primer	1.5	20 µM	600 nM
Reverse Primer	1.5	20 µM	600 nM
QIAGEN OneStep RT-PCR Enzyme	0.2	-	-
Nuclease-free water	28.0	-	-
TOTAL VOLUME	**50 µl**	**-**	**-**

* Contains 12.5 mM MgCl2

The GeneAmp 2400 Thermal Cycler (Perkin-Elmer, UK) was programmed with separate conditions for the F gene and G gene to accommodate the T_m of the primers (Table 6.3).

Table 6.3. Thermal cycling conditions for the OneStep RT-PCR Kit.

The annealing conditions differed for the fusion (top) and glycoprotein (bottom) primer pairs to accomodate the differences in T_m of the primers.

Step	Temperature	Time	Cycles
Reverse transcription:	50°C	30 minutes	1 cycle
Initial PCR activation step:	95°C	15 minutes	1 cycle
3-step cycling			
Denaturation:	94°C	30 seconds	
Annealing:	58°C	60 seconds	40 cycles
Extension:	72°C	60 seconds	
Final Extension:	72°C	10 minutes	1 cycle

Step	Temperature	Time	Cycles
Reverse transcription:	50°C	30 minutes	1 cycle
Initial PCR activation step:	95°C	15 minutes	1 cycle
3-step cycling			
Denaturation:	94°C	60 seconds	
Annealing:	55°C	60 seconds	40 cycles
Extension:	72°C	60 seconds	
Final Extension:	72°C	10 minutes	1 cycle

The annealing temperature of thermal cycling conditions for the G gene was decreased incrementally if no bands were visible in the agarose gel (Table 6.4). The reaction tubes were placed on ice until the thermal cycler reached 50°C. Finally, the reaction tubes were loaded into the chamber of thermal cycler. After thermocycling, the cDNA was stored at 2°C to 8°C overnight or at -20°C for prolonged storage.

Table 6.4. Thermal cycling conditions for the OneStep RT-PCR Kit.

The annealing temperature of thermal cycling conditions for the G gene was decreased incremenatally if no bands were visible in the agarose gel.

Step	Temperature	Time	Cycles
Reverse transcription:	50°C	30 minutes	1 cycle
Initial PCR activation step:	95°C	15 minutes	1 cycle
3-step cycling			
Denaturation:	94°C	60 seconds	
Annealing:	55°C	60 seconds	5 cycles
Extension:	72°C	60 seconds	
Denaturation:	94°C	60 seconds	
Annealing:	50°C	60 seconds	5 cycles
Extension:	72°C	60 seconds	
Denaturation:	94°C	60 seconds	
Annealing:	45°C	60 seconds	30 cycles
Extension:	72°C	60 seconds	
Final Extension:	72°C	10 minutes	1 cycle

6.3.6. Purification of amplification products

Amplification products were purified using the QIAquick® PCR purification kit (QIAGEN Ltd, Crawley, West Sussex, UK) to remove excess nucleotides and enzyme contamination from DNA fragments that would otherwise interfere with subsequent downstream applications according to manufacturer's instructions (Section 3.3).

6.3.7. Sequence determination

Sequencing reactions were performed by Genome Enterprise Limited, The Genome Analysis Centre, Norwich Research Park, Norwich, UK using the gene specific forward and reverse primers described in section 6.3.2 in combination with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) on the 3730*xl* DNA Analyser (Applied Biosystems, Warrington, UK).

6.3.8. Sequence analysis and alignment

All F and G gene sequences downloaded from GenBank® were analysed altogether (unrooted). Pairwise and multiple nucleotide sequence alignments were generated with ClustalW software. Sequences were viewed using Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0 (Tamura *et al.*, 2011) or Geneious Pro™ software version 5.4 (Drummond *et al.*, 2011). Phylogenetic trees were constructed from 1000 samplings of

maximum composite likelihood (MCL) distances by neighbour-joining method with pair-wise deletions for missing nucleotides in MEGA 5 (Tamura *et al*., 2011).

6.3.9. Statistical Analysis

Statistical calculations were performed with SPSS version 17.0 (SPSS Inc, Chicago, Illinois, USA). A *p* value of <0.05 was considered statistically significant. To test the hypothesis that HMPV strains circulating within the locality of Norwich differed from other strains within a sublineage, a one-way analysis of variance (ANOVA) was used for the comparison of continuous variables.

6.4. Results

6.4.1. Analysis of the fusion and glycoprotein protein genes of human metapneumovirus

In total, 36 NPA samples collected between 31st October 2005 and 31st December 2008, which were positive for HMPV by real-time RT-PCR targeting the N gene of the virus were analysed by a conventional end-point RT-PCR assay targeting two independent regions of the HMPV F gene ORF and G gene ORF using the same nucleic acid eluate. The PCR products, which were analysed using 2% pre-cast agarose gels were then identified as the correct size using a Quantitative DNA Ladder (Figure 6.1 and 6.2). Overall, 100% of samples were positive by the end-point RT-PCR assay targeting the F gene ORF while 86.1% samples were positive for the G gene ORF target sequence. The sensitivity and specificity of the gene-specific primer pairs were not evaluated in the present study. No extraneous bands were visualised on the agarose gels inferring specificity of the primer pairs but the absence of a discrete band corresponding to the 807 bp fragment of the G gene suggests that the conventional end-point RT-PCR assay failed to amplify the target sequence from nucleic acid extracted from five NPA samples. The additional intervention of incrementally decreasing the annealing temperature of thermal cycling conditions for the G gene primer pair failed to provide a resolution to the absent PCR product. Real-time RT-PCR was performed using primers and TaqMan® probe designed to target the HMPV N gene prior to the conventional end-point RT-PCR assay with the G gene and F gene primer pairs to ensure the positive status of all clinical samples. The fluorescent signal produced for some samples was weak and it is plausible that this contributed to the unsuccessful amplification of the respective target sequence. However, since all 36 samples were identified using the F gene primer pair it is more likely that the primers targeting the G gene ORF failed to amplify the target sequence.

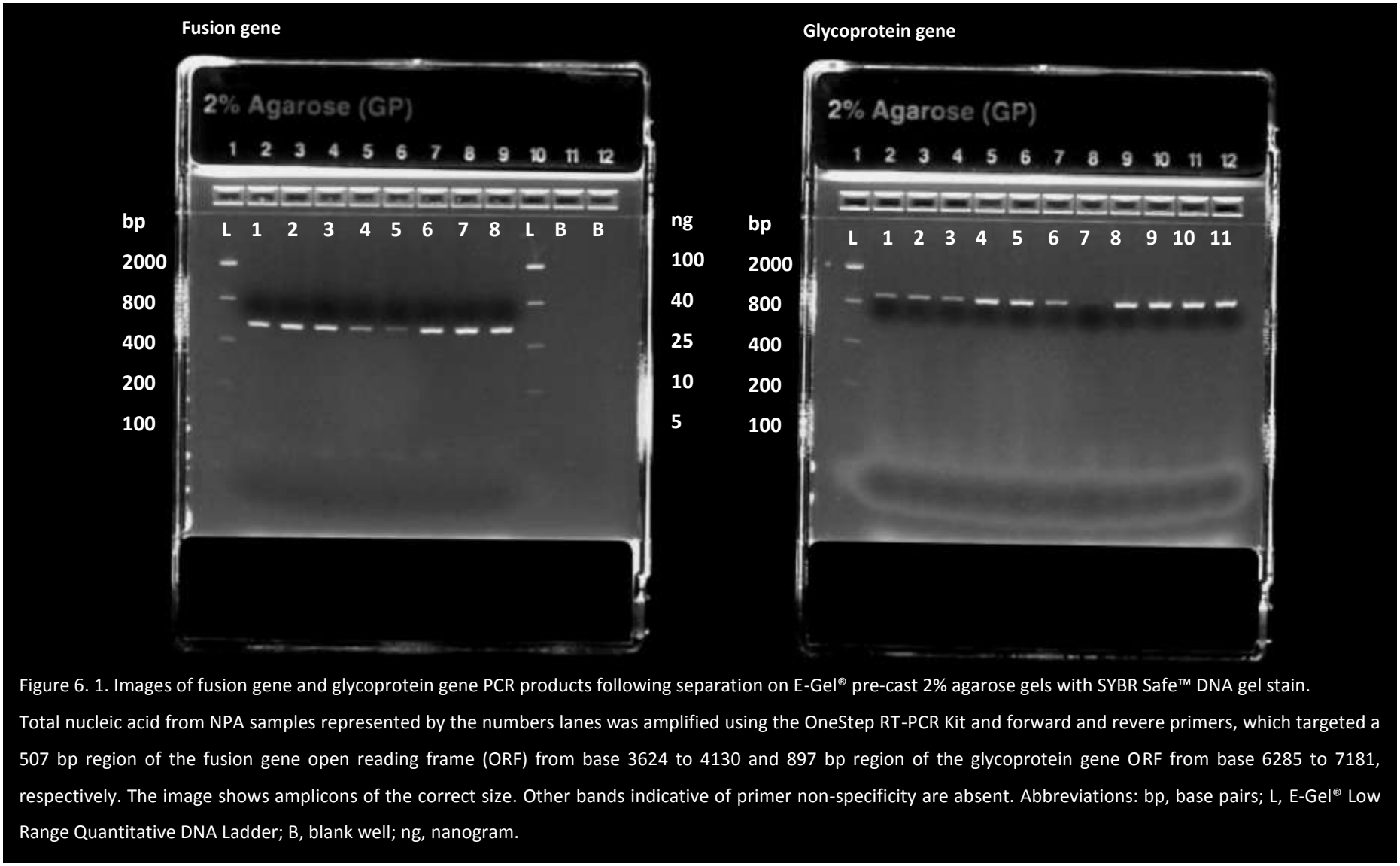


Figure 6. 1. Images of fusion gene and glycoprotein gene PCR products following separation on E-Gel® pre-cast 2% agarose gels with SYBR Safe™ DNA gel stain. Total nucleic acid from NPA samples represented by the numbers lanes was amplified using the OneStep RT-PCR Kit and forward and revere primers, which targeted a 507 bp region of the fusion gene open reading frame (ORF) from base 3624 to 4130 and 897 bp region of the glycoprotein gene ORF from base 6285 to 7181, respectively. The image shows amplicons of the correct size. Other bands indicative of primer non-specificity are absent. Abbreviations: bp, base pairs; L, E-Gel® Low Range Quantitative DNA Ladder; B, blank well; ng, nanogram.

6.4.2. Phylogenetic analysis of HMPV Fusion and Glycoprotein Gene Sequences

The molecular epidemiology of HMPV was examined by phylogenetic analysis of partial nucleotide sequences within the F gene ORF and G gene ORF of HMPV strains circulating among a cohort of paediatric patients within the locality of Norwich during a three-year period together with all publicly available F and G gene sequences within the NCBI nucleotide database. This revealed the major HMPV genetic lineages, A and B, which were further divided into sublineages A1 and A2 (Figure 6.2) and B1 and B2 (Figure 6.3). The A2 sublineage was further bi-partitioned into two distinct clusters representing A2a and A2b (Figure 6.2). Sequences from HMPV positive samples collected from paediatric patients attending the Norfolk and Norwich University Hospital were assigned to genetic lineage A and B and further partitioned into sublineages A2a, A2b, B1, and B2; no sequences within the sublineage A1 were identified. Genetic lineage distribution between partial F and G gene sequences was identical for mutual HMPV strains circulating within the cohort. The phylogenetic tree generated from partial G gene sequences showing the partitioning of genetic lineages A and B is shown in Figure 6.2 and Figure 6.3, respectively. The existence of the two major genetic lineages, A and B, and sublineages A1, A2, B1, and B2 was strongly supported by bootstrap analysis. Lineage A and B were separated in 100% of bootstrap replicas while sublineages A1 and A2, B1, and B2 were separated in 95-100% of bootstrap replicas. The estimated nucleotide identities between strains circulating within the locality of Norwich are displayed within a pairwise identity heatmap (Figure 6.4). The same sublineage appears as blocks shaded black while different sublineages appear as blocks shaded palest gray indicative of high and low sequence homology, respectively. The estimated percentage nucleotide identities for the G gene between the two major lineages, A and B were 51.3% - 54.8% but higher percentage identities were observed within sublineages (Table 6.5). Unfortunately, only one strain belonging to sublineage B1 was identified within the cohort so it was not possible to determine the nucleotide identities within sublineage B1.

Table 6.5. Percentage nucleotide identities for the glycoprotein gene of predominant strains of human metapneumovirus circulating within a cohort of paediatric patients within the locality of Norwich between 31st October 2005 and 31st December 2008..

	Percentage (%) nucleotide identities			
Sublineages	A2a	A2b	B1	B2
A2a	91.8-97.4	83.1-87.6	53.3-54.8	53.3-58.0
A2b		87.4-95.7	51.8-53.1	51.8-56.5
B1			N/A	72.7-75.1
B2				86.7-98.0

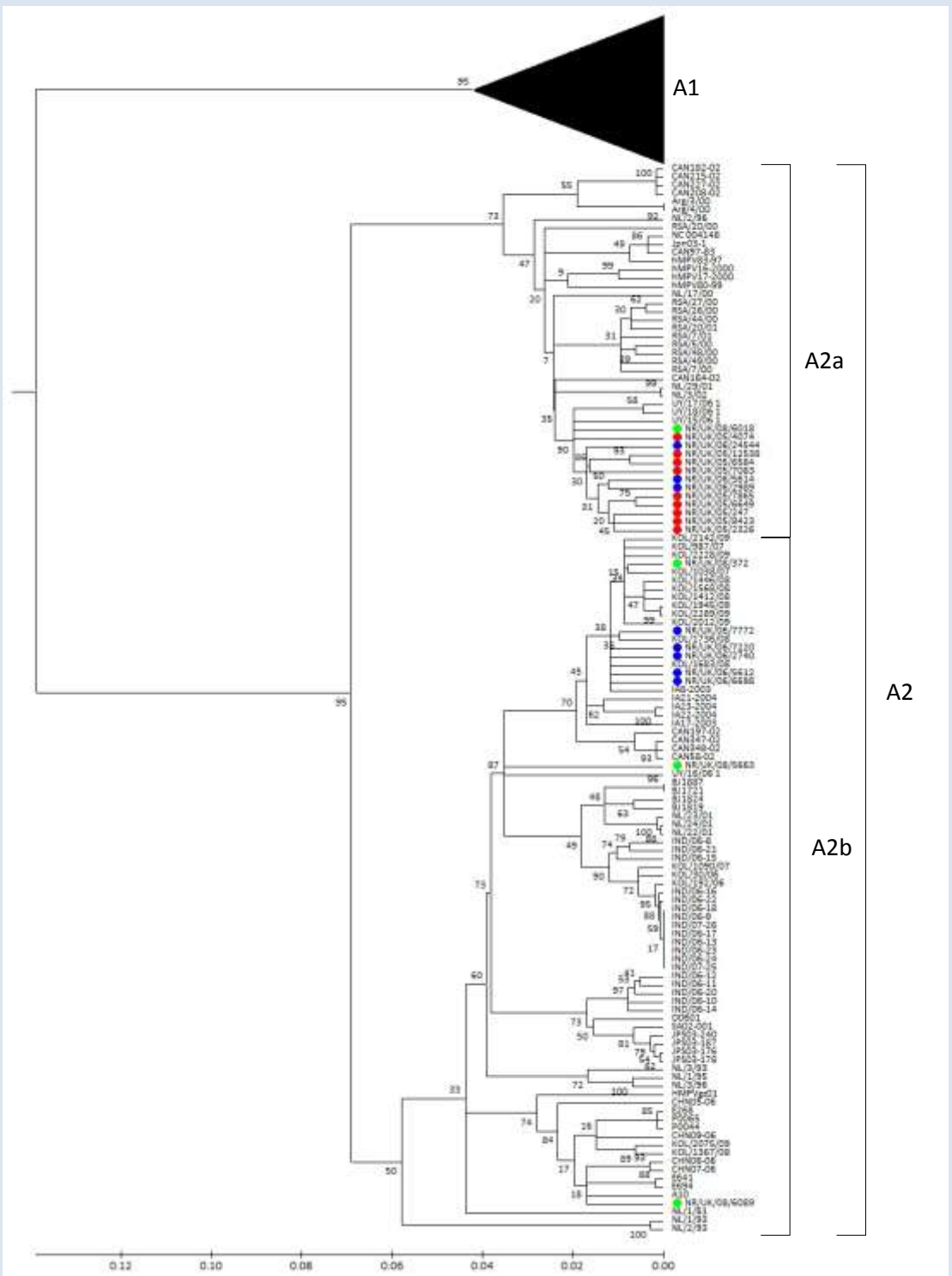


Figure 6.2. Phylogenetic trees of partial nucleotide sequences of the glycoprotein gene of human metapneumovirus showing genetic sublineages A1, A2a, and A2b.

The phylogenetic tree was constructed in Molecular Evolutionary Genetics Analysis software version 5.0 (Tamura *et al.*, 2011) using the neighbour-joining method with 1000 bootstrap replicates from partial glycoprotein gene sequences available from the National Center for Biotechnology Information database and sequences identified within the present study during 2005 (●), 2006 (●), and 2008 (●). Genetic lineage A1 is represented as a compressed subtree as no sequences within this lineage were identified within the present study.

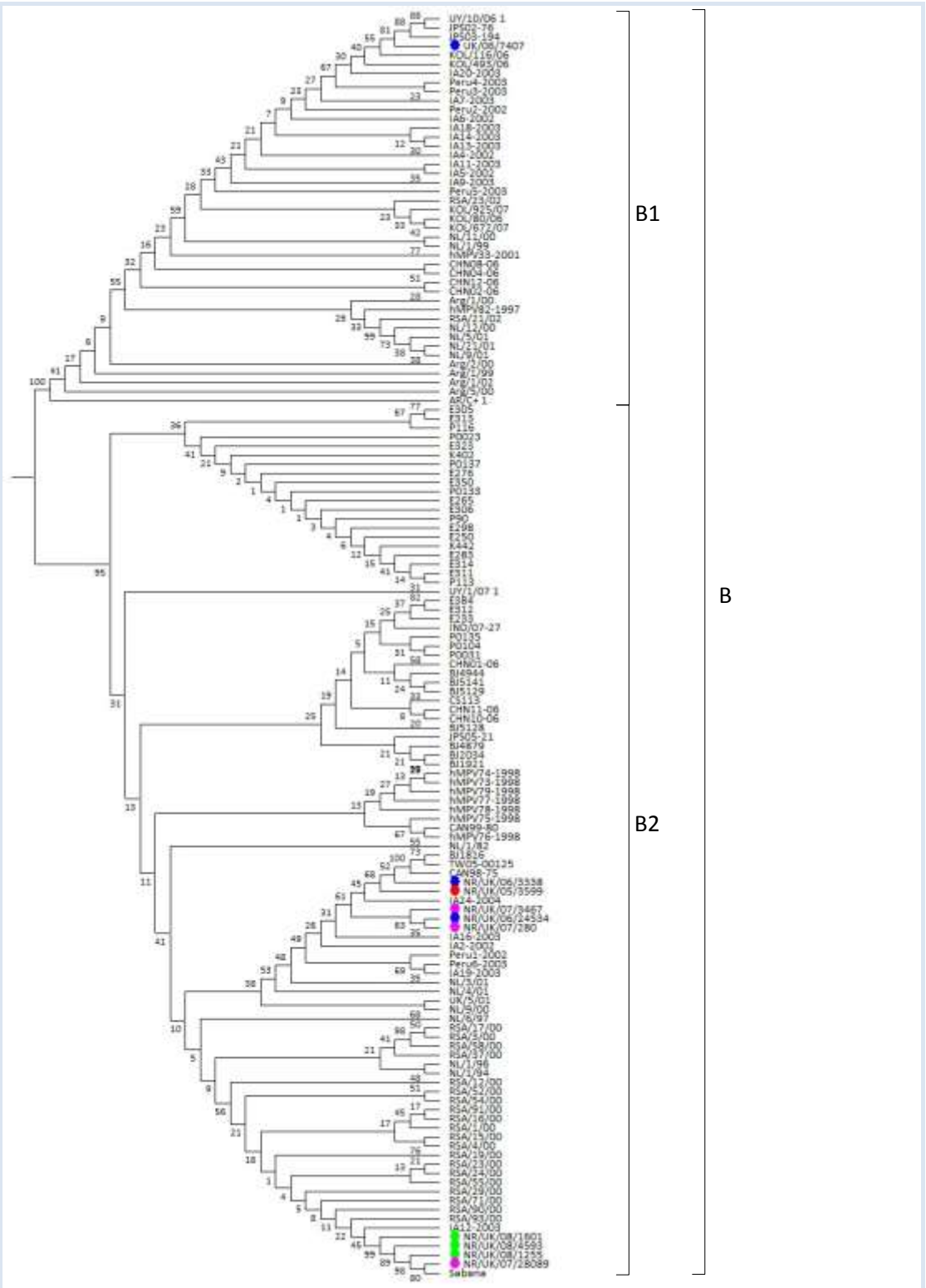


Figure 6.3. A phylogenetic tree of partial nucleotide sequences of the glycoprotein gene of human metapneumovirus showing genetic sublineages B1 and B2.

The phylogenetic tree was constructed in Molecular Evolutionary Genetics Analysis software version 5.0 (Tamura *et al.*, 2011) using the neighbour-joining method with 1000 bootstrap replicates from partial glycoprotein gene sequences available from the National Center for Biotechnology Information database and sequences identified within the present study during 2005 (●). 2006 (●). 2007. (●). and 2008 (●).

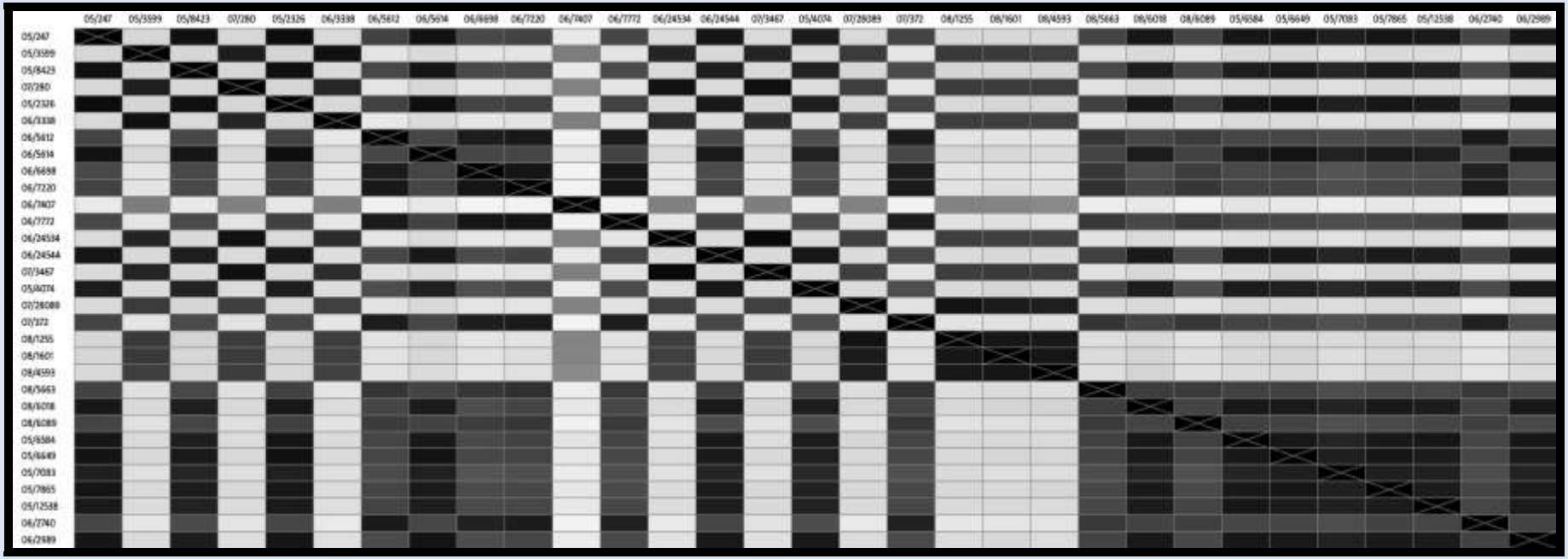


Figure 6.4. A pairwise nucleotide identity heatmap displaying sequence homology between strains of human metapneumovirus circulating amongst a cohort of paediatric patients within the locality of Norwich.

A pairwise nucleotide sequence alignment for all 31 glycoprotein gene sequences was performed using Geneious Aligner™ within Geneious Pro™ softare version 5.4 (Drummond *et al*., 2011). Sequence identity between each pair of sequences was measured. The heatmap was generated using Geneious Pro™ software version 5.4 (Drummond *et al*., 2011) as a two-dimensional graphical representation of the values of the pairwise distance matrix.

6.4.3. Seasonal variability of HMPV lineages and sublineages

The two major genetic lineages, A and B, co-circulated among the cohort of paediatric patients each epidemiological year, which was defined as May 1st to April 30th of the following year. Lineage A remained dominant throughout the study period with 61.1% (22/36) of all strains circulating within the cohort belonging to this lineage. Lineage A predominated in 2004/2005 and 2005/2006 whilst circulation of lineage B remained constant but an increase in circulation of lineage B during epidemiologic year 2007/2008 arose following a decline in lineage A (Figure 6.5). A switch in predominance occurred again in 2008/2009 with the increase in circulation of lineage A.

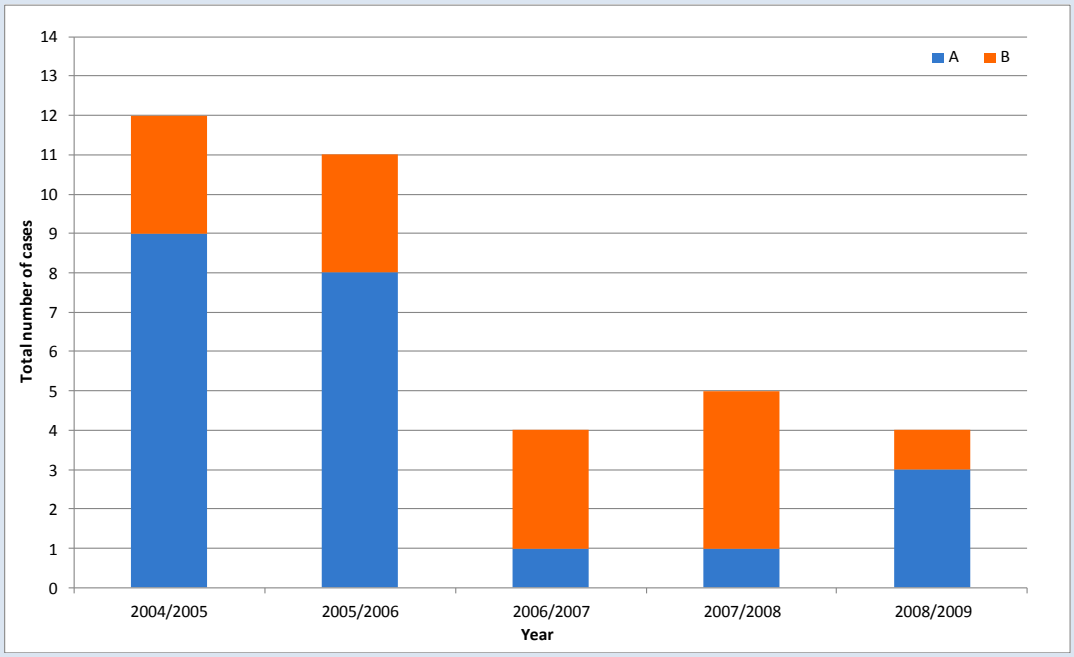


Figure 6.5. Distribution of the two distinct genetic lineages of human metapneumovirus, A and B, circulating among a cohort of paediatric patients within the locality of Norwich each epidemiological year.

The partitioning of lineages, A and B, is based on phylogenetic analysis of partial fusion gene sequences.

The distribution of the genetic sublineages A2a, A2b, B1, and B2 varied each respiratory season (Figure 6.6). Sublineage A2a established dominance throughout the three-year period with 14/36 (38.9%) strains circulating within the cohort belonging to this sublineage followed by sublineages B2 (n= 11; 30.6%), A2b (n= 8; 22.2%) and finally B1 (n= 3; 8.3%) (Figure 6.6). There was a shift in dominance from sublineage A2a in 2004/2005 to A2b in 2005/2006 following a decline in circulation of sublineage A2a and a corresponding surge in circulation of sublineage A2b. Both sublineage B1 and B2 circulated among the paediatric

cohort in 2004/2005 and 2005/2006 but sublineage B1 disappeared entirely from circulation in subsequent epidemic years with a corresponding increase in circulation of sublineage B2. A further transferral of dominance to sublineage B2 was observed in 2006/2007, which continued in 2007/2008. Sublineage B2 was superseded by sublineage A2b in 2008/2009 as the dominant circulating sublineage (Figure 6.6).

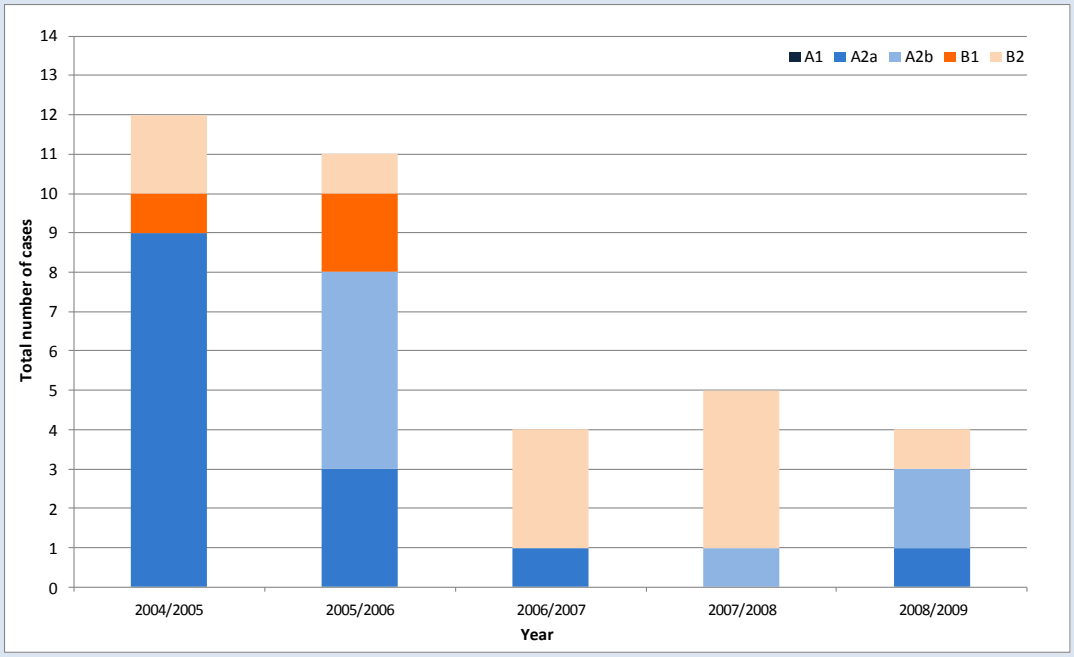


Figure 6.6. Phylogenetic evolution of human metapneumovirus among a cohort of paediatric patients within the locality of Norwich by epidemiological year.

The repartition of human metapneumovirus between genetic sublineages A2, B1, and B2 is displayed for each epidemiologic year. The partitioning of sublineages, A2, B1, and B2, is based on phylogenetic analysis of partial fusion gene sequences.

HMPV sublineage A2a emerged as the primary cause of HMPV infection in January 2005. A surge in circulation of sublineage A2a was observed within the cohort during March 2005 (Figure 6.7). Sublineage A2a receded in April 2005 but continued to circulate within the cohort during February and March 2006 albeit at a much reduced level and emerged again for a final appearance in December 2006 before disappearing entirely until December 2008 (Figure 6.7). Sublineage A2b emerged in February 2006 with a peak in circulation in April 2006, which represented a potential second outbreak of HMPV infection. This lineage disappeared from circulation until a fleeting appearance in January 2008 (Figure 6.7). Sublineage B1 and B2 continued to circulate at a low level with sublineage A2a and A2b throughout the winter-spring seasons 2004/2005 and 2005/2006, respectively but a switch to sublineage B2 dominance arose, which appeared to correspond with disappearance of sublineage A2 from circulation. The last episode of HMPV infection associated with

sublineage B2 occurred in October 2008 and was followed by a surge in circulation of sublineage A2 in December 2008 (Figure 6.7).

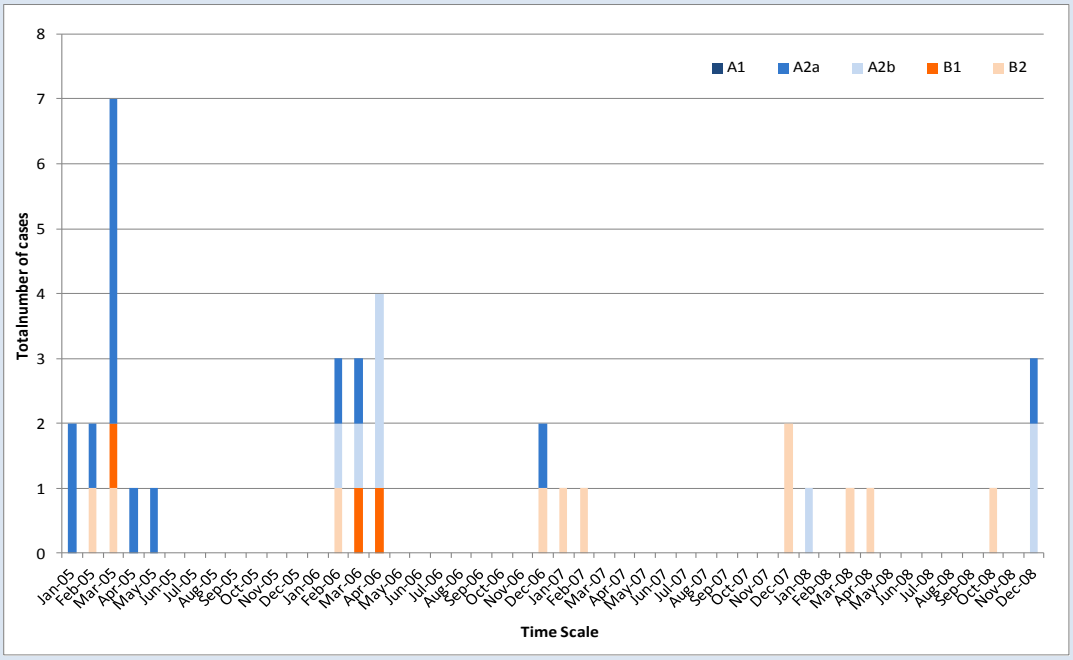


Figure 6.7. The repartition of human metapneumovirus between sublineages A2, B1, and B2 among a cohort of paediatric patients within the locality of Norwich is displayed each month during the three-year period investigated. The partitioning of sublineages, A2, B1, and B2, is based on phylogenetic analysis of partial fusion gene sequences.

Interestingly, the majority of HMPV strains within sublineage A2a circulating among the cohort within the locality of Norwich were clustered tightly together and were generally distinct from most other strains within this sublineage (Figure 6.8). A one-way ANOVA was undertaken to test the hypothesis that the majority of HMPV strains within sublineage A2a circulating within the locality of Norwich represented a distinct subcluster.

Table 6.6. A one-way ANOVA was undertaken to test the hypothesis that the majority of HMPV strains within sublineage A2a circulating within the locality of Norwich represented a distinct subcluster. A *p* value of <0.05 was considered statistically significant.

	SS	df	MS	F	Significance of ratio *p* value
Between Groups	628.6975	2	314.3488	5.736949	0.006
Within Groups	2246.542	41	54.79372		
Total	**2875.24**	**43**			

Abbreviations: SS, Sum of Squares; df, degrees of freedom; MS, Mean Square; F; ratio of the mean squares.

The one-way, between-subjects analysis of variance revealed that HMPV strains clustered within sublineage A2a circulating within the locality of Norwich represent a distinct subcluster (Table 6.6). Consequently, it appears that a novel strain or closely related strains within sublineage A2a caused a large outbreak within the paediatric cohort in 2005 and continued to contribute to smaller outbreaks in subsequent years.

Comparison of HMPV strains circulating amongst the paediatric cohort with strains within the NCBI public database also reveals the longevity and wide geographical distribution of several strains belonging to sublineages A2b (Figure 6.2), B1 and B2 (Figure 6.3) circulating within the locality of Norwich.

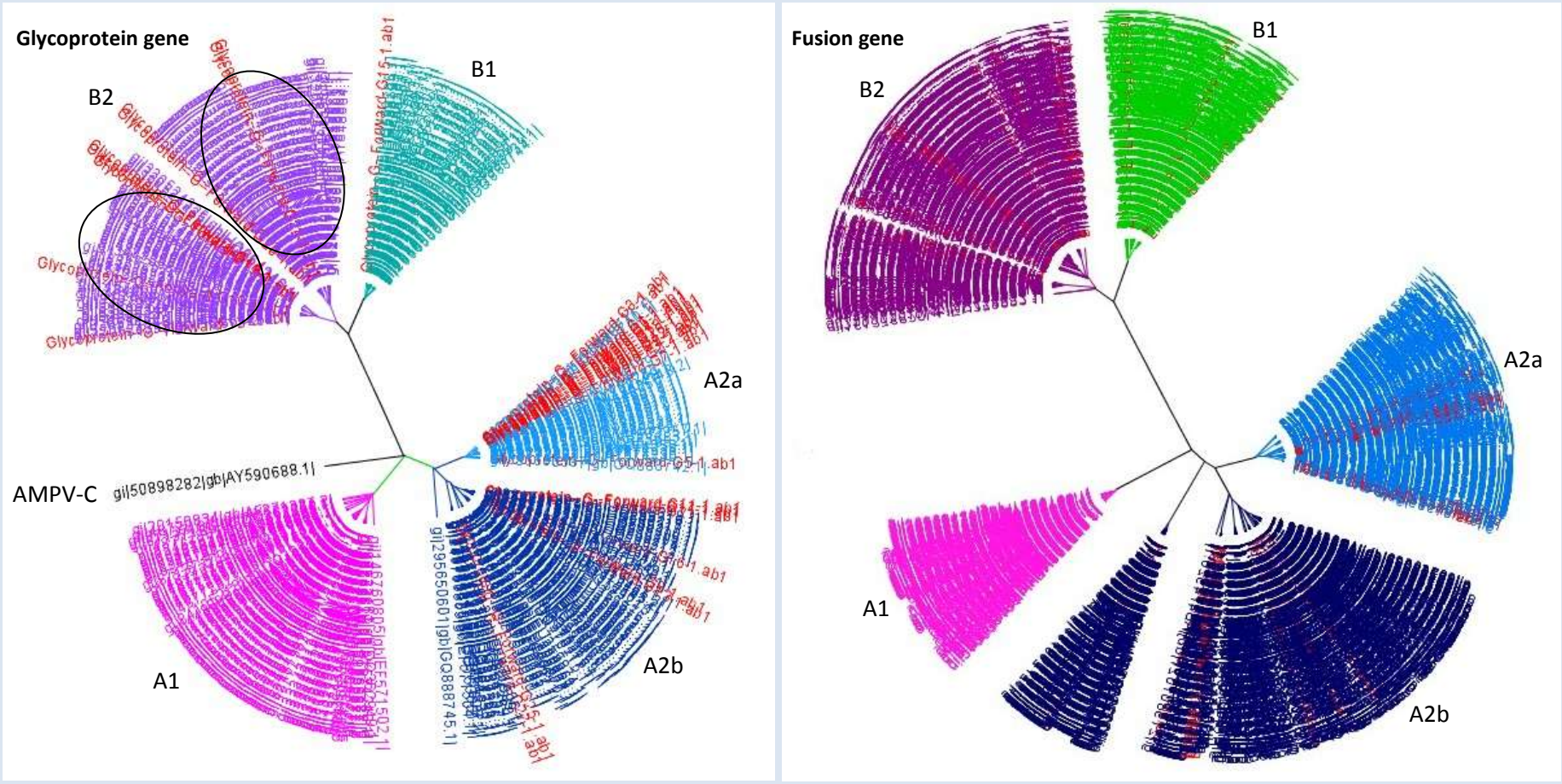


Figure 6.8. Phylogenetic trees of nucleotide sequences of the glycoprotein gene and fusion gene of human metapneumovirus showing the genetic lineages and sublineages of the virus.

The phylogenetic trees were constructed in Geneious Pro™ software version 5.4 (Drummond *et al*., 2011) using the neighbour-joining method with 1000 bootstrap replicates from partial glycoprotein and fusion gene sequences available from the National Center for Biotechnology Information database and sequences identified within the present study during 2005, 2006, 2007, and 2008 (●). The outbreak of human metapneumovirus within the paediatric cohort is clearly visible within the phlyogenetic tree of partial nucleotide sequences of the gycoprotein gene and fusion gene. Abbreviations: AMPV-C, avian metapneumovirus.

6.5. Discussion

The present study provides support to existing phylogenetic analysis conducted within the highly variable G gene (Ludewick *et al*., 2005; van den Hoogen *et al*., 2004b) and conserved regions of the HMPV genome including the N (Gerna *et al*., 2005; Huck *et al*., 2006), P (Mackay *et al*., 2004), and F genes (Galiano *et al*., 2006; Gerna *et al*., 2005; Huck *et al*., 2006; Ludewick *et al*., 2005; Oliveira *et al*., 2009) for the years 2005 to 2008. Epidemiologic surveillance and molecular biology are slowly revealing the complex and dynamic epidemiology (Kahn, 2006) of this ubiquitous respiratory pathogen. Global co-circulation of genetic sublineages, A1, A2, B1, and B2, within a single year is not unusual (Aberle *et al*., 2010; Carr *et al*., 2008; Gerna *et al*., 2005; Huck *et al*., 2006; Mackay *et al*. 2006; Sloots *et al*., 2006). Both major genetic lineages, A and B, co-circulated among the cohort within the locality of Norwich but sublineages, A1, A2, B1, and B2 failed to continue the trend established by the parental lineages. Nevertheless, multiple sublineages circulated within the cohort each epidemiologic year. Many of the strains circulating among the paediatric cohort were clustered within the subclusters, A2a and A2b, within sublineage A2 (Huck *et al*., 2006). Recently, Carr *et al*., (2008) described two new genetic subclusters within sublineage B2, referred to as B2a and B2b. There has been limited acknowledgement of the further bipartition of sublineage B2 in contrast to sublineage A2. However, two subclusters within sublineage B2 are clearly discernible within the phylogenetic tree of partial nucleotide sequences of the G gene (Figure 6.8).

The absence of sublineage A1 within the present cohort is echoed within studies conducted in years 2006 to 2010 (Agrawal *et al*., 2011; Arnott *et al*., 2011b; Carr *et al*., 2008; Banerjee *et al*., 2011; Gaunt *et al*., 2009; Lamson *et al*., 2012; Li *et al*., 2012; Mizuta *et al*., 2010). However, sublineage A1 circulated widely within the human population between 1982 and 2005 (Aberle *et al*., 2010; Hopkins *et al*., 2008; Gerna *et al*., 2005; Mackay *et al*. 2006; Wang *et al*., 2008; Williams *et al*., 2006b) and dominated the circulation hierarchy in most studies conducted in years 2000, 2001, and 2002 (Aberle *et al*., 2010; Gerna *et al*., 2005; Legrand *et al*., 2011; Ludewick *et al*., 2005; Mackay *et al*. 2006; Sloots *et al*., 2006), a finding that suggests that specific strains may co-exist across geographic areas in a given epidemic (Ludewick *et al*., 2005). Whilst sublineage A1 was displaced from predominance by the emergence of sublineage B1 in the Southern Hemisphere (Ludewick *et al*., 2005; Mackay *et al*. 2006; Sloots *et al*., 2006) parallel studies in the Northern Hemisphere report differences in the predominant circulating subtype (Aberle *et al*., 2010; Gerna *et al*., 2005). It is unclear whether the predominance of sublineage B1 within the Southern Hemisphere was a

coincidental finding or a spatial and temporal anomaly. Certainly, geographic and temporal distinction is associated with HRSV (Choi and Lee, 2000). Discrimination of potential trends confounded by conflicting nomenclature (Hopkins *et al*., 2008) and inconsistent classification of genetic lineages, which conceals the genetic variability exhibited by the virus (Huck *et al*., 2006). Furthermore, limited studies present detailed extended longitudinal evidence of the molecular epidemiology of this virus (Aberle *et al*., 2010) and although studies invariably overlap gaps remain between studies conducted over different time scales (Agrawal *et al*., 2011; Carr *et al*., 2008; Gerna *et al*., 2005; Huck *et al*., 2006; Ludewick *et al*., 2005; Oliveira *et al*., 2009; Pitoiset *et al*., 2010; Pizzorno *et al*., 2010; Sloots *et al*., 2006; Wang *et al*., 2008). Furthermore, the majority of the studies were conducted in the Northern Hemisphere and few data exist from extended studies over several years that involve populations in other parts of the world (Oliveira *et al*., 2009).

Although, the present study was restricted to a single geographical region it was possible to determine some parallel changes within the UK and Ireland. Carr *et al*., (2008) evaluated the genetic diversity of HMPV circulating within hospitalised children <5 years presenting with respiratory tract infection in Dublin, Ireland between March and May 2006 and November and February 2007, which revealed sublineages A2 and B2 circulating within the population. Sublineage A2b dominated in 2005/2006 while B2 dominated in 2006/2007 akin to the present study. In another study, Gaunt *et al*., (2009) screened respiratory samples collected between 1 July 2006 and 30 June 3008. Sublineage A2b predominated in 2006/2007 in contrast to the circulation pattern observed in Dublin and Norwich. However, a switch to sublineage B2 dominance in 2007/2008 united the circulation patterns observed within England, Ireland, and Scotland suggesting that other factors influence HMPV circulation. In fact, Gaunt *et al*., (2009) included respiratory samples collected from all age groups within hospital and primary care settings, which suggests that HMPV circulation within distinct communities may be highly variable like HRSV (Arnott *et al*., 2011a) but also that similar strains cycle through adjacent geographic areas (Larcher *et al*., 2008). Nevertheless, evidence for the longevity and wide geographical distribution of HMPV lineages and sublineages (Gaunt *et al*., 2009) is provided by strains circulating within the paediatric cohort, which resemble strains circulating globally. Sublineage A2b strains resembled strains circulating in India (Agrawal *et al*., 2011) and Uruguay (Pizzorno *et al*., 2010) whilst B1 and B2 strains were similar to strains circulating in Japan (Ishiguro *et al*., 2004) and China (Liu *et al*., 2010), Taiwan (Huang *et al*. 2010), and Canada (Biacchesi *et al*., 2003), respectively. The identification of very similar strains present simultaneously in widely geographical regions is

described for HRSV, suggesting that outbreak strains may spread globally (Peret *et al*., 1998). It is plausible that HMPV strains from different geographical regions can be introduced into a community, but then local factors, such as population patterns of strain-specific immunity, determine which of these strains circulate efficiently and become 'outbreak strains' as is suspected to occur with respect to HRSV (Peret *et al*., 1998).

The displacement of the predominant circulating sublineage in successive epidemics is a habitual finding (Chung *et al*., 2008; Gaunt *et al*., 2009; Huck *et al*., 2006; Mackay *et al*. 2006; Pitoiset *et al*., 2010) but is not universal to all studies including the present one (Galiano *et al*., 2006; Sloots *et al*., 2006). Aberle *et al*., (2010), demonstrated this episodic change in predominant circulating sublineage most succinctly during a 21-year period. Within this study, one sublineage predominated each year, but was displaced by another sublineage every 1 to 3 years (Aberle *et al*., 2010). Many human infecting viruses undergo a turnover and replacement of predominant lineages with emergent strains (Gaunt *et al*., 2011). Indeed, consistent shifts in the predominant genotype or subtype over successive epidemics is a phenomenon exhibited by HRSV, which confers a significant advantage to the virus since the 'novel' virus is better able to evade previously induced immunity in the population and consequently either circulates more efficiently or is more pathogenic (Cane *et al*., 1994; Peret *et al*., 1998; Seki e*t al*., 2001). Similarly, it is hypothesised that switching of the predominant circulating HMPV lineage is brought about by short-lived lineage-specific herd immunity in a population generated over one or two seasons, which favours dissemination of the alternate lineage in a subsequent season (Gaunt *et al*., 2011).

Two distinct serotypes corresponding to the two major genetic lineages of HMPV, A and B, were defined on the basis of sequence diversity between attachment protein genes and differences between homologous and heterologous virus neutralisation titres in sera obtained from ferrets infected with HMPV belonging to different genetic sublineages (van den Hoogen et al., 2004b). Higher virus neutralisation titres were demonstrated against homologous virus than heterologous virus within the neutralisation assays (van den Hoogen et al., 2004b). Likewise, cross-challenge experiments aimed at inducing antibodies against the conserved HMPV F protein in animal models revealed that primary infection with either of the two main genetic lineages of HMPV induced protective immunity against subsequent challenge with homologous or heterologous virus (MacPhail *et al*., 2004; Skiadopoulos *et al*., 2004; van den Hoogen *et al*., 2007). Together these experimental studies demonstrate that the F protein is a major antigenic determinant that mediates extensive cross-lineage

neutralisation and protection (Skiadopoulos *et al*., 2004). Conversely the glycoproteins G and SH play minor or insignificant roles in stimulating neutralisation and protection (Biacchesi *et al*., 2005). It is postulated that antigenic variability of the G gene may play an important role in the ability of this virus to escape the pre-existing immune response (Kahn, 2006) and a selective advantage for heterologous virus during subsequent epidemics, even in the presence of the broadly cross-reactive anti-F humoral immunity in the population (Schildgen *et al*., 2011). Genetic variability is a strong indicator of positive selection and affects the ability of a virus to continue circulating in a population (Ludewick *et al*., 2005). The HMPV G gene is highly variable, particularly in the extracellular domain, because of nucleotide substitutions, insertions, and the use of alternative termination transcription codons (Ludewick *et al*., 2005). In a recent study, positively selected sites found for the G protein were located in the extracellular domain and corresponded to potential *O*-linked glycosylated sites (de Graaf *et al*., 2008). It is known that simian immunodeficiency virus (SIV), HIV, influenza virus, HCV, Ebola virus, and porcine reproductive and respiratory syndrome virus (PRRSV) rely on glycosylation modification of envelope proteins to evade the host immune response (Vu *et al.*, 2011). Evidence suggests that HMPV may too utilise this mechanism to shelter the virus from immunologic recognition (Bastien *et al*., 2004), which may explain the transient nature of the anti-G antibody response (Endo *et al*., 2008) and the high rate of re-infection observed in all age groups throughout life, which facilitates frequent outbreaks of infection in vulnerable populations including children, the immunocompromised, and the elderly (Boivin *et al*., 2007; Døllner *et al*., 2004; Heikkinen *et al*., 2008; Honda *et al*., 2006; Kim *et al*., 2009; Liao *et al.,* 2011; Louie *et al*., 2007; Omura *et al*., 2011; Osbourn *et al*., 2009; Pabbaraju *et al*., 2007) despite high seroprevalence rates (Lüsebrink *et al*., 2010). Furthermore, inhibition of IFN signalling may help explain why long-term protective immunity is not seen with HMPV infection (Dinwiddie and Harrod, 2008). Proper signalling by IFN-α and IFN-γ is vital for clearance of viral pathogens because of their immunoregulatory functions that affect both innate and adaptive immunity (Dinwiddie and Harrod, 2008). Thus, inhibition of IFN signalling by HMPV may alter the host's ability to develop proper adaptive immunity leaving the host susceptible to re-infection (Dinwiddie and Harrod, 2008). Ultimately, further detailed extended longitudinal studies of the molecular epidemiology of this virus are required that extend to all parts of the world in order to gain a better understanding of the genetic heterogeneity of circulating HMPV lineages. This will allow the further resolution of the HMPV G protein including identification of other sites under positive selection, which perhaps contribute to the observed differential disease severity caused by different HMPV lineages. Certainly, the recent identification of

two epitopes within the central conserved region of the HRSV G protein ectodomain showed that the region between amino acids 149 and 177 played no role in virus infectivity. It is predicted that the loss of these residues may therefore reduce virus immunogenicity while having no effect on virus infectivity. These observations together with previous reports of premature stop codons and frame shifts within the G protein of HRSV subgroup B suggest that this subgroup may use quite different mechanisms to evade host immune responses (Gaunt *et al*., 2011). It seems that the differential location of positive selected sites within the HMPV G protein of different HMPV lineages may also occur in keeping with the observations made of HRSV (Gaunt *et al*., 2011).

Phylogenetic data from Edinburgh indicates re-circulation of strains over at least 8 years (Gaunt *et al*., 2009). It is postulated that strains may circulate within a community during an epidemic season, migrate to evade induced immunity and return when transient anti-glycoprotein immunity has waned (Gaunt *et al*., 2009). Similarly, circulation of sublineage A2a waned and eventually disappeared in 2006/2007 after an outbreak of HMPV infection within the paediatric cohort during epidemiologic year 2004/2005 only to reappear in epidemiologic year 2008/2009. Unfortunately, the re-emergence of sublineage A2a corresponded with end of the study period so it was not possible to determine whether sublineage A2a became dominant once more although sublineage A2a predominated in other regions in the Northern Hemisphere during the 2009/2010 winter-spring season including the United States (Lamson *et al*., 2012) and South Korea (Kim *et al*., 2012). Interestingly, recent evidence suggests that sustained circulation of contemporary HMPV lineages for decades and the global dissemination of the virus demonstrate that switching of the predominant genetic group did not arise through the emergence of novel lineages each respiratory season, but through the fluctuating circulation frequencies of pre-existing lineages, which undergo proliferative and eclipse phases. Proliferation occurs when the lineage is of minimal susceptibility to the adaptive immune responses of the host population, and a regression in circulating frequency occurs as the host population is increasingly exposed. During the eclipse phase, the virus evolves immune evasive characteristics, which when accumulated sufficiently permit a new phase of widespread circulation (Gaunt *et al*., 2011).

Phylogenetic analysis of partial G gene sequences revealed a unique strain or closely related strains within sublineage A2a circulating within the locality of Norwich, which was responsible for a large outbreak of infection within the paediatric cohort in epidemiologic

year 2004/2005. Phylogenetic analysis of F gene sequences also revealed this distinct subcluster within sublineage A2a although it is clustered with other strains, which suggests closer similarities to other strains than apparent from analysis of the G gene sequences. This might be a reflection of the genetic variability of the G gene or increased utilisation of the F gene for molecular surveillance because of difficulties in amplifying regions within the G gene (Arnott *et al*., 2011b; Pizzorno *et al*., 2010; van den Hoogen *et al*., 2004b). Boivin *et al*., (2004) highlighted that primer design for the G gene selected from the sequence of the prototype strain for lineage A1, NL/1/00 (GenBank accession no. AF371337) might not be optimal for successful amplification of strains belonging to lineage B. The primer pair selected for detection of the G gene ORF within this study was designed from NL/1/00 (Ludewick *et al*., 2005) providing a possible explanation for failure to successfully amplify the target sequence from 5 NPA samples, which were identified as belonging to lineage B by phylogenetic analysis of F gene sequences.

CHAPTER SEVEN

7. Concluding remarks and future research needs

It has been 10 years since HMPV was identified as a causative agent of respiratory illness in humans (Schildgen *et al*., 2011). The anniversary was marked by the publication of two review articles celebrating the achievements in the understanding of this novel respiratory virus over the past decade (Schildgen *et al*., 2011; Feuillet *et al*., 2011). The discovery of HMPV has influenced many of the developments in the diagnosis of respiratory virus infection. The inability to diagnose HMPV infection by traditional diagnostic methods compelled many diagnostic laboratories to reconsider the clinical utility of existing diagnostic tests and offered an opportunity to promote the need for new technology and scientific tools (Chapter 3). Nucleic acid amplification techniques soon became the method of choice for HMPV detection owing to the resolution of intrinsic limitations associated with traditional methods and were introduced increasingly within the diagnostic setting reflecting the clinical demand for routine HMPV testing (Hopkins *et al*., 2008).

This work commenced soon after the discovery of HMPV with the development of an internally controlled one-step real-time RT-PCR assay for the diagnosis of HMPV infection, which signalled the start of the expansion of the clinical virology diagnostic service within the Microbiology Department (Chapter 3). Ultimately, the requirements of the clinical virology diagnostic service have served to shape as well as limit the scope of research undertaken. Nevertheless, work presented here reiterates the contribution of HMPV to the burden of respiratory disease within the paediatric population, which accounted for 4.6% of acute respiratory infections previously not attributed to known respiratory pathogens in hospitalised children during the 3 year period investigated (Chapter 4). Moreover, the prevalence of the virus across categories of age and gender and the seasonal and annual distribution pattern of HMPV in contrast to other well-established respiratory viruses were in agreement with previous studies, which offered clarification of the epidemiological characteristics of the virus amongst this population within the UK as well as the rest of the world (Chapter 4). These findings provided justification for the implementation of routine HMPV testing, which has facilitated public health surveillance and supported patient management and the prevention and control of infection within the hospital setting.

The introduction of newer multiplexed methodologies, which permit the simultaneous detection and identification of multiple infectious agents in acute-phase respiratory specimens (Brunstein *et al*., 2008) has further revolutionised the diagnosis of respiratory

virus infections within the routine diagnostic setting (Chapter 5). Retrospective evaluations of newer multiplexed methodologies including the xTAG® RVP test and a panel of 3 in-house multiplex real-time RT-PCR assays revealed that the existing combination of virus culture, DFA and NOW® RSV Test failed to diagnose approximately 50% of respiratory infections attributed to new and well-established respiratory viruses (Chapter 5). This startling revelation further supported the change to sensitive molecular systems for the diagnosis of respiratory virus infections. The evaluation also revealed that multiple infections are frequent and provided further evidence for complex interactions between different infectious agents (Brunstein *et al*., 2008) (Chapter 5). No associations were observed between HMPV and other respiratory viruses within this study, which reiterates that the majority of HMPV infections are not associated with other viruses (Schildgen *et al*., 2011) (Chapter 4). However, significant negative associations were identified between other respiratory viruses convincingly demonstrated in previous studies (Chapter 5), which in light of the restricted sample size investigated provides further import to the idea that associations do not occur by chance (Greer *et al*., 2009). Interest in polyviral infections is an emergent area within the discipline of virology aided by the introduction of newer multiplex molecular methods. Whilst few studies have acknowledged specific patterns between viruses as something more than a simple overlap of epidemic seasons there is growing evidence for complex interactions between viruses present in co-detections. The work presented here offers a significant and important contribution to limited studies conducted hitherto through substantiation of newly recognised associations between viruses present in co-detections. Certainly, the suggestion that HRV infection can act an inadvertent natural moderator of other viral infections (Greer *et al*., 2009) may in this new role, re-write our understanding of commensal flora and viral respiratory infections (Chapter 5). Nevertheless, interpreting the mechanisms underlying these observations of virus interference is complex (Wisdom *et al*., 2009). It is not easy to determine whether such co-infections are sequential or simultaneous viral infections (Söderlund-Venermo *et al.,* 2009), and if sequential, what is the order of acquisition of these infections (Wisdom *et al*., 2009)**.** This unknown factor may significantly contribute to the outcome of polyviral interactions (Wisdom *et al*., 2009) and elucidate differences between studies in relation to the clinical severity of HMPV infection. Moreover, this missing link may offer insight for the disparity in the association between HMPV and HRV observed here in contrast to an earlier study (Greer *et al*., 2009). As a novel area of investigation, further studies are required to clarify the nature of the interaction between these viruses, which may have important future implications since HRV acquisition may reduce the risk of HMPV infection and thus, indirectly, the spread of the virus. Future

studies should aim to illuminate the role of HRV in viral interference whilst no prophylaxis or vaccine exists against either HMPV or HRV, which may alter the interaction between this virus pair before we can fully decipher it.

Even fewer studies have considered the importance of bacterial co-infection in HMPV-associated RTI. While a direct interaction between *S. pneumoniae* and HMPV infection is suggested evidence is thus so far lacking to form a complete picture of the relationship between HMPV and other bacteria habitually implicated in mixed viral-bacterial respiratory infections despite the well documented importance of respiratory viruses in facilitating secondary bacterial infections. HMPV was identified more commonly with one or more concomitant respiratory bacteria than as a sole respiratory pathogen or in combination with other viruses within the present study (Chapter 4). These results suggest the direct involvement of HMPV in the development of bacterial-viral co-infections and reiterate that the pathogenesis of HMPV-associated acute respiratory infection frequently involves bacterial co-infection (Madhi *et al*., 2006). Both *H. influenzae* and *S. pneumoniae* were found frequently with HMPV in NPA samples, either as a solitary companion to HMPV or in combination with other bacterial pathogens. However, only *H. Influenzae* was significantly associated with HMPV infection (Chapter 4). While previous studies have implicated HMPV in the pathogenesis of pneumococcal pneumonia, no direct correlation was evident between *S. pneumoniae* and HMPV in the present study. Instead, a positive correlation with the presence of *H. influenzae* and HMPV was found. These unique findings may reflect complex changes in the epidemiology of *S. pneumoniae* since the introduction of the PCV7 in the England and Wales. Current evidence suggests that exposure to *S. pneumoniae* can influence susceptibility to HMPV infections (Madhi *et al*., 2006; Verkaik *et al*., 2011) (Chapter 4). However, *in vivo* studies were conducted in populations where vaccines to control *S. pneumoniae* were not currently part of the immunisation programme (Verkaik *et al*., 2011) or in use in a specific population (Madhi *et al*., 2006) while *in vitro* studies do not take into consideration complex microbial community interactions (Kukavica-Ibrulj *et al*., 2009; Verkaik *et al*., 2011) (Chapter 4). These findings serve to reiterate that the prevalence of pathogens within the human population is changing, and often to an extreme degree, as a direct effect of medical practices and lifestyle changes (Blaser and Falkow, 2009). While the impact of some of these changes is now appreciated, it is plausible that the correlation between HMPV and *H. influenzae* represents a previously unreported repercussion of the use of this vaccine to control *S. pneumoniae* infection in children (Chapter 4). A comprehensive investigation is required in order to determine the full extent to which these

changes have influenced the relationship between *S. pneumoniae* and other commensal flora of the respiratory tract that interact with respiratory viruses. Moreover, consideration of potential changes within the microbial communities of other age groups is currently lacking despite the finding that herd immunity to pneumococcal vaccine serotypes has been induced in the UK population as an indirect effect of infant PCV7 immunisation (Gladstone *et al*., 2011) with decreases in all vaccine serotypes in all age groups (Miller *et al*., 2011). Hence, it would be interesting to determine whether the positive association between HMPV and *H*. *influenzae* is observed in other age groups. The ability to characterise accurately bacterial communities may be crucial if pathogenesis is related to changes in community composition (Rogers *et al*., 2009). It would seem imprudent to ignore the effect that social and medical progress is having on the composition of the human microbiota, as it seems that there may be a price to pay for the lack of appreciation of these changes. A greater understanding of the characteristics of a host's genome and microbiota, and their interactions, will lead to individualised approaches to the prevention and treatment of specific diseases (Blaser and Falkow, 2009). It is hoped that the work presented here will make a valuable contribution to the understanding of HMPV and particularly in relation to the role of vaccination on relationships between respiratory viruses and common bacterial pathogens and that the points raised will open discussion on the implications of vaccination on mixed viral-bacterial respiratory infections.

The human microbiome, in general, is assumed beneficial to the host due to stimulation and maturation of immune systems, promotion of mucosal structure and function and providing actual 'colonisation resistance' against pathogen invasion (Bogaert *et al*., 2011). However, by creating an imbalance in the composition of microbiota does vaccination alter the stimulation and maturation of the immune system and so increase the ability of a virus to create a niche for other pathogenic organisms to colonise? This paradigm may offer a new outlook to explain the variability in the severity of disease observed with HMPV. Obviously, answers to these questions are beyond the scope of the present study but it is clear that there is much to understand in relation to this virus and the discovery of previously unknown relationships suggests that a major re-appraisal is required of the role of viral and bacterial pathogens in the development of respiratory infection. Such is the importance of this work that ideally studies would be collaborative and conducted at a global level. Future studies should examine a much larger data set of respiratory specimens for multiple pathogens to enable a more thorough interpretation of the results presented here. It is imperative that diagnostic methods start to generate a complete picture of infection rather than as we have

presently a rather incomplete and possibly distorted one (Rogers *et al*., 2009). Newer multiplexed methods will allow direct comparisons of the prevalence of multiple respiratory pathogens, which in parallel with clinical data from case-controlled studies should further illuminate the clinical significance of these interactions. Perhaps future projects centred on the human microbiome might consider characterisation of viruses relative to microbial communities within the human nasopharyx, which would offer a universal view of the dynamic relationships between bacteria and viruses.

Chronic bacterial infections of the lower airways and specifically those that occur in cystic fibrosis patients represent an ideal model system for investigating the processes that are involved in the development and dynamics of polymicrobial infections (Rogers *et al*., 2009) while the naive lung of young children is offering the opportunity to observe the evolution of resident microbiota before exposure to influences that lead to the imbalance in its composition (Bogaert *et al*., 2011). Consideration must also be given to genetic factors that have the potential to influence our susceptibility to the nasopharyngeal microbiota as well as viruses such as polymorphisms in the structural and promoter sequences of the mannose-binding lectin (MBL) gene, which affect the assembly of this complex molecule and interfere with its complement activating function (Eisen, 2010). Ultimately, the ability to characterise accurately the cause of infection is fundamental to effective treatment. Molecular approaches that assess the content of clinical samples promise to change dramatically the types of data that are obtained routinely from clinical samples. In addition to the technical advance that these methodologies offer, a conceptual advance in the way that we reflect on the information generated is also required. Through the development of both of these advances, our understanding of infection, as well as the ways in which infections can be treated, may be improved (Rogers *et al*., 2009).

Phylogenetic analysis approaches are helping to improve our understanding of the nature of HMPV respiratory infections. Within the present study, phylogenetic analysis of sequences within the highly variable G gene and conserved F gene was conducted in order to determine the relative genetic variability of all known HMPV lineages circulating within the paediatric cohort within the locality of Norwich (Chapter 6). Unfortunately, the present study was only conducted over a 3-year period, which provided limited opportunity to discern the molecular epidemiology of the virus. However, in combination with other studies it seems a pattern is emerging of the circulation frequencies of the two distinct genetic lineages of HMPV despite the paucity of longitudinal studies. It is clear that many aspects of the molecular

epidemiology of HMPV and HRSV exist in parallel. Indeed, these ubiquitous respiratory viruses are counterparts in many ways and the analogies between these viruses have provided a greater understanding of HMPV. In agreement with other studies conducted to investigate the molecular epidemiology of HMPV, the findings presented in this study suggest that HMPV exhibits local and global circulation of both genetic lineages akin to HRSV with switching of the predominant circulating sublineage every 1 to 3 years (Chapter 6). Additionally, the data suggests that a unique strain circulated within the paediatric cohort within the locality of Norwich, which suggests local factors influence HMPV circulation although these are yet to be determined but may include population patterns of strain-specific immunity. Future extended longitudinal studies conducted in worldwide collaboration are required to determine the long-term and spatial circulation trends of predominant virus lineages. Such studies will be essential to the understanding of differences in clinical severity between the two major genetic lineages, A and B. Investigations should include the causative aetiology of respiratory infection inclusive of viral and bacterial pathogens using consistent methodologies and ideally based on sensitive molecular methodologies in order to determine the full infection picture, which may also influence disease severity.

The last decade has witnessed a major contribution toward the understanding of HMPV but much remains to be discovered. The molecular epidemiology of the virus and the consequent persistent evasion of a sustained immune response remains the biggest conundrum. Certainly, the implications of the circulation of two serotypes of HMPV have raised uncertainty regarding the viability of an effective vaccine since early in the discovery of the virus (van den Hoogen *et al*., 2004b). For HRSV, the importance of difference in antigenicity between the two subgroups regarding protective immunity and vaccine development is still a subject of discussion, which provides an indication of the difficulties yet to come in the production of a vaccine for HMPV. Perhaps those waiting for a safe and effective HRSV vaccine may question the validity of vaccine development for HMPV when there is still no effective vaccine for HRSV. However, the importance of HMPV and HRSV in the development of severe infections in the immunocompromised, very young, and frail elderly hosts emphasises the need for effective vaccines, particularly within the developing world, where supportive therapy is not available (Lindell *et al*., 2011).

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APPENDICES

Norfolk (1) Research Ethics Committee
c/o The Norfolk & Norwich University Hospital NHS Trust
First Floor
Aldwych House
57 Bethel Street
Norwich
NR2 1NR

Telephone: 01603 289813
Facsimile: 01603 286573

10 August 2005

Miss Lindsay Butcher
Clinical Scientist Grade B
Microbiology Department
Norfolk & Norwich University Hospital NHS Trust
Bowthorpe Road
Norwich
NR2 3TX

Dear Miss Butcher

Full title of study: **The contribution of human metapneumovirus to respiratory tract disease**
REC reference number: **05/Q0101/77**

The REC gave a favourable ethical opinion to this study on 13 June 2005.

Further notification(s) have been received from local site assessor(s) following site-specific assessment. On behalf of the Committee, I am pleased to confirm the extension of the favourable opinion to the new site(s). I attach an updated version of the site approval form, listing all sites with a favourable ethical opinion to conduct the research.

Management approval
The Chief Investigator or sponsor should inform the local Principal Investigator at each site of the favourable opinion by sending a copy of this letter and the attached form. The research should not commence at any NHS site until management approval from the relevant NHS care organisation has been confirmed.

Statement of compliance
The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

05/Q0101/77 **Please quote this number on all correspondence**

Yours sincerely

Mrs Janette Guymer
Committee Co-ordinator
Email: janette.guymer@nnuh.nhs.uk

An advisory committee to Norfolk, Suffolk and Cambridgeshire Strategic Health Authority

Enclosure:

List of Sites with Favourable Ethical Opinion - Site approval form (SF1)

Copy to:

Louise Briggs
Research Contracts Manager
Research & Business Services
The Registry
University of East Anglia
Norwich NR4 7TJ

Norfolk 1

LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.

REC reference number:	05/Q0101/77	**Issue number:**	2	**Date of issue:**	10 August 2005
Chief Investigator:	Miss Lindsay Butcher				
Full title of study:	The contribution of human metapneumovirus to respiratory tract disease				

This study was given a favourable ethical opinion by Norfolk1 on 13 June 2005. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.

Principal Investigator	*Post*	*Research site*	*Site assessor*	*Date of favourable opinion for this site*	*Notes* [1]
Miss Lindsay Jayne Butcher	Clinical Scientist Grade B	The Norfolk and Norwich University Hospital NHS Trust	Norfolk (1)	16/06/2005	East Norfolk & Waveney RGC Ref 2005MICRO15
"	"	University of East Anglia	Norfolk (1)	10/08/2005	"

Approved by the Chair on behalf of the REC:

..... (Signature of ~~Chair~~/Administrator)

(delete as applicable)

J R GUYTER (Name)

[1] *The notes column may be used by the main REC to record the early closure or withdrawal of a site (where notified by the Chief Investigator or sponsor), the suspension of termination of the favourable opinion for an individual site, or any other relevant development. The date should be recorded.*

Norwich Research Ethics Committee
c/o The Norfolk & Norwich University Hospital NHS Trust
First Floor
Aldwych House
57 Bethel Street
Norwich
NR2 1NR

Telephone: 01603 286 397
Facsimile: 01603 286 573

16 June 2005

Miss Lindsay Butcher
Clinical Scientist
Microbiology Department
Norfolk & Norwich University Hospital NHS Trust
Bowthorpe Road
Norwich
NR2 3TX

Dear Miss Butcher

Full title of study: **The contribution of human metapneumovirus to respiratory tract disease**
REC reference number: **05/Q0101/77**

The Research Ethics Committee would wish to thank you for attending the meeting held on 13 June 2005 to discuss the above application.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation.

Following clarification at the meeting that laboratory work will be carried out at the UEA Members request that an application for a Site Specific Assessment for that site is submitted for review.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Application	4.1: Parts A,B & C	17 May 2005
Investigator CV		(None Specified)
CV	Supervisor - Professor Paul Hunter	(None Specified)

Protocol	1	11 March 2005
Covering Letter		20 May 2005
Peer Review	EN&W Research Governance Committee	09 May 2005

Management approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final management approval from the R&D Department for the relevant NHS care organisation.

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Notification of other bodies

The Committee Administrator will notify the research sponsor and the R&D Department for NHS care organisation(s) that the study has a favourable ethical opinion.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

05/Q0101/77 Please quote this number on all correspondence

With the Committee's best wishes for the success of this project,

Yours sincerely

Dr Elizabeth Lund
Vice Chair

Enclosures:

Attendance at Committee meeting on 13 June 2005
Standard approval conditions
Site approval form (SF1)

Norwich Research Ethics Committee

LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.

REC reference number:	05/Q0101/77	**Issue number:**	1	**Date of issue:**	15 June 2005
Chief Investigator:	Miss Lindsay Butcher				
Full title of study:	The contribution of human metapneumovirus to respiratory tract disease				

This study was given a favourable ethical opinion by Norwich Research Ethics Committee on 16 June 2005. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.

Principal Investigator	*Post*	*Research site*	*Site assessor*	*Date of favourable opinion for this site*	*Notes (1)*
Miss Lindsay Jayne Butcher	Clinical Scientist	Norfolk and Norwich University Hospital NHS Trust	Norwich Research Ethics Committee	16/06/2005	2005MICR01S

Approved by the Chair on behalf of the REC:

..... (Signature of Chair/Administrator)
(delete as applicable)

..... (Name)

(1) *The notes column may be used by the main REC to record the early closure or withdrawal of a site (where notified by the Chief Investigator or sponsor), the suspension of termination of the favourable opinion for an individual site, or any other relevant development. The date should be recorded.*

Phosphate Buffered Saline (Dulbecco `A' Tablets)

Phosphate buffered saline (PBS) was prepared by dissolving 10 tablets (Oxoid Ltd, Hampshire, UK) in 1 litre of double distilled water. The buffer was sterilised by autoclaving at 115^{0}C for 10 minutes at a pressure of 1.1 bar (15 Ib/in^2 or 15 psi) and stored at room temperature (15^{o}C to 25^{o}C).

4 M Sodium Hydroxide (NaOH)

A 4 M NaOH solution was prepared by adding 160 g NaOH (Sigma-Aldrich, Dorset, UK) to 1 litre of double distilled water.

10X Tris-Borate-EDTA (TBE) buffer

To prepare a 10X TBE solution, the contents of 1 package of 10X crystalline TBE (Ambion®, Warrington, UK) was combined with 1 L of double distilled water until completely dissolved.

1X Tris-Borate-EDTA (TBE) buffer

To prepare a 1X working solution containing 89 mM Tris, 89 mM Borate, and 2 mM EDTA, 100 ml of 10X TBE solution was diluted with 1 L of double distilled water.

10% Glucose

A 10% glucose solution was prepared by adding 10 g of anhydrous D(+)-glucose (VWR International Ltd, Dublin, Ireland) to 100 ml of double distilled water. The solution was filter sterilised through a 0.22 µm sterile 33 mm Millex-GP filter unit (Millipore, Watford, UK) and stored at 2^{o}C to 8^{o}C in 10 ml aliquots.

1 M calcium chloride ($CaCl_2$)

A 1 M calcium chloride solution of calcium chloride ($CaCl_2$) was prepared by adding 14.701 g of $CaCl_2$ (Sigma-Aldrich, Dorset, UK) to 100 ml of double distilled water. The solution was filter sterilised through a 0.22 µm sterile 33 mm Millex-GP filter unit (Millipore, Watford, UK) and stored at 2^{o}C to 8^{o}C in 10 ml aliquots.

Thiamine hydrochloride (10 mg/ml)

A 10 mg/ml solution of thiamine hydrochloride was prepared by adding 10 g of thiamine hydrochloride (Sigma-Aldrich, Dorset, UK) to 1 L of double distilled water. The solution was filter sterilised through a 0.22 µm sterile 33 mm Millex-GP filter unit (Millipore, Watford, UK) and stored at 2^{o}C to 8^{o}C in 10 ml aliquots wrapped in foil to protect from light.

Kanamycin solution (25 mg/ml)

A 25 mg/ml stock solution of kanamycin was prepared by adding 2.5 g of kanamycin sulphate (Sigma-Aldrich, Dorset, UK) to 100 ml of double distilled water. The solution was filter sterilised through a 0.22 µm sterile 33 mm Millex-GP filter unit (Millipore, Watford, UK) and stored at -20^{o}C in 5 ml aliquots.

Broth Medium #271

Broth medium #271 was prepared by dissolving 10 g of tryptone powder (Oxoid Ltd, Hampshire, UK), 1.0 g of yeast extract powder (Oxoid Ltd, Hampshire, UK), 8.0 g of sodium chloride (NaCl) (VWR International Ltd, Dublin, Ireland) in 1 litre of double distilled water. Once combined the medium was autoclaved at 121^{0}C for 15-20 minutes at a pressure of 1.1 bar (15 lb/in^2 or 15 psi). The medium was cooled to approximately 50^{0}C before aseptically adding 10 ml of 10% glucose, 2.0 ml of 1M $CaCl_2$ and 1.0 ml of 10 mg/ml thiamine hydrochloride. The medium was stored at 2^{o}C to 8^{o}C for not more than 1 month prior to use.

Solid Agar Underlay

Solid agar was prepared by dissolving 10 g of tryptone powder (Oxoid Ltd, Hampshire, UK), 1.0 g of yeast extract powder (Oxoid Ltd, Hampshire, UK), 8.0 g of NaCl (VWR International Ltd, UK), and 15 g of Agar Bacteriological (Agar No 1.) (Oxoid Ltd, Hampshire, UK) in 1 litre of distilled water. Once combined the medium was autoclaved at 121^{0}C for 15-20 minutes at a pressure of 1.1 bar (15 ib/in^2 or 15 psi). The molten agar was cooled to approximately 50^{0}C before aseptically adding 10 ml of 10% glucose, 2ml of 1M $CaCl_2$ and, 1 ml of 10 mg/ml thiamine hydrochloride. The molten agar was poured into petri-dishes and allowed to solidify at room temperature (15 ^{o}C to 25^{o}C). The plates were inverted once the agar was set to avoid condensate that formed on the lid from falling onto the agar surface. A nutrient agar slant was prepared by transferring 5 ml of molten agar to a 10 ml sterile glass universal. The universal was retained at a 45^{o} angle whilst the medium solidified. All solid media were stored at 2^{o}C to 8^{o}C for not more than 1 month prior to use.

Soft Agar Overlay

Soft agar to overlay the solid agar was prepared by dissolving 1.0 g of tryptone powder, 0.1 g of yeast extract, 0.8 g of NaCl, 0.5 g of Difco™ Noble Agar (BD Diagnostics, Oxford, UK) in 100 ml of distilled water. Once combined the medium was autoclaved at 121^{0}C for 15-20 minutes at a pressure of 1.1 bar (15 Ib/in^2 or 15 psi). The medium was stored at 2^{o}C to 8^{o}C for not more than 1 month prior to use.

Luria-Betani (LB) medium

LB medium was prepared by dissolving 10 g of Bacto™ Tryptone (BD Diagnostics, Oxford, UK), 5.0 g of Bacto™ Yeast Extract (BD Diagnostics, Oxford, UK), 10 g of NaCl (VWR International Ltd, UK) in 950 ml of double distilled water. Once combined, the pH of the medium was adjusted to pH 7 with dropwise additions of NaOH (Appendix II) before the volume was made up to 1 litre with double distilled water. The medium was poured into 100 ml glass bottles with loosened caps and autoclaved at 121^{0}C for 25 minutes at a pressure of 1.1 bar (15 lb/in^2 or 15 psi). The molten medium was cooled to <50^{0}C to prevent excessive heat from degrading the antibiotic before aseptically adding 50 µl of 100 mg/ml ampicillin ready-made solution (Sigma-Aldrich, Dorset, UK) to give a final concentration of 50 µg/ml. Alternatively, 200 µl of 25 mg/ml kanamycin solution (Sigma-Aldrich, Dorset, UK) (Appendix II) was added to the molten medium to give a final concentration of 50 µg/ml. LB medium was stored at room temperature (15^{o}C to 25^{o}C).

LB agar plates

LB agar was prepared by dissolving 10 g of Bacto™ Tryptone (BD Diagnostics, Oxford, UK), 5.0 g of Bacto™ Yeast Extract (BD Diagnostics, Oxford, UK), 10 g of NaCl (VWR International Ltd, UK), and 15 g of Bacto™ Agar (BD Diagnostics, Oxford, UK) in 950 ml of double distilled water. Once combined, the pH of the medium was adjusted to pH 7 with dropwise additions of NaOH before the volume was made up to 1 litre with double distilled water. The medium was poured into 100 ml glass bottles with loosened caps and autoclaved at 121^{0}C for 25 minutes at a pressure of 1.1 bar (15 lb/in^2 or 15 psi). The molten agar was cooled to ~50^{0}C to prevent excessive heat from degrading the antibiotic before aseptically adding 50 µl of 100 mg/ml ampicillin ready-made solution (Sigma-Aldrich, Dorset, UK) to give a final concentration of 50 µg/ml. Alternatively, 200 µl of 25 mg/ml kanamycin solution (Sigma-Aldrich, Dorset, UK) (Appendix II) was added to the molten agar to give a final concentration of 50 µg/ml. The molten agar was poured into sterile petri-dishes using approximately 40 ml per plate and allowed to solidify at room temperature (15^{o}C to 25^{o}C). The plates were

inverted once the agar was set to avoid condensate that formed on the lid from falling onto the agar surface. The plates were stored at 4^{o}C.

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL)

A 40 mg/ml stock solution of X-GAL (Melford Laboratories Ltd, Suffolk, UK) was prepared by dissolving 40 mg of X-GAL in 1 ml of *N, N*-Dimethylformamide (DMF) (Sigma-Adrich, Dorset, UK). The solution was stored at -20^{o}C wrapped in foil to protect from light.

2x Yeast-Tryptone (YT) Microbial Medium

2x YT microbial medium is a standard nutrient media for maintenance and propagation of recombinant strains of *Escherichia coli*. It was prepared by dissolving 16 g of Bacto™ Tryptone (BD Diagnostics, Oxford, UK), 10 g of Bacto™ Yeast Extract (BD Diagnostics, Oxford, UK), and 5g of NaCl (VWR International Ltd, UK) in 1 litre of double distilled water. The medium was autoclaved at 121^{0}C for 25 minutes at a pressure of 1.1 bar (15 lb/in^{2} or 15 psi). 2x YT microbial medium was stored at room temperature (15 ^{o}C to 25^{o}C).

Primer and probe sequences that comprise the real-time quadriplex reverse-transcription polymerase chain reaction assay for the detection of influenza viruses.

Residue M represents an adenine (A) or a cytosine (C) residue, residue Y is either a C or a thymidine (T) residue, residue W is either an A or T residue, and residue R is either an (A) or a guanine (G) residue.

Primer or probe	Sequence (5'-3')	Target gene
Forward Primer	GCC GAA TGA TGC MAT MAA YT	Haemagglutinin
Reverse primer	CGC ACC CAT TGG AGT TTG AC	
Probe	6-FAM – CAT TGC TCC AGA AWA T – MGBNFQ	

Primer and probe sequences for influenza A virus subtype H5. The TaqMan® minor groove binder (MGB™) probe was labelled at the 5' end with the fluorescent reporter dye 6-carboxyfluorescein (6-FAM) and a non-fluorescent quencher (NFQ) at the 3' end.

Primer or probe	Sequence (5'-3')	Target gene
Forward primer	GAG TCT TCT AAC MGA GGT CGA AAC GTA	Matrix
Reverse primer	GGG CAC GGT GAG CGT RAA	
Probe	VIC – TCC TGT CAC CTC TGA C – MGBNFQ	

Primer and probe sequences for influenza virus type A. The TaqMan® MGB™ probe was labelled at the 5' end with the fluorescent reporter dye VIC® and a NFQ at the 3' end. VIC® is a proprietary dye only availble from Applied Biosystems.

Primer or probe	Sequence (5'-3')	Target gene
Forward primer	GCA GCT CTG ATG TCC ATC AAG CT	Nucleoprotein
Reverse primer	CAG CTT GCT TGC TTA RAG CAA TAG GTC T	
Probe	Cy5 – CCA GAT CTG GTC ATT GGR GCC CAR AAC TG – BHQ-3	

Primer and probe sequences for influenza virus type B. The probe was labelled at the 5' end with the fluorescent reporter dye Cyanine (CY)-5 and a non-fluorescent quencher, Black Hole Quencher™ 3 (BHQ-3) at the 3' end.

Primer or probe	Sequence (5'-3')	Target gene
Forward primer	TGG CAC TAC CCC TCT CCG TAT TCA CG	3' end
Reverse primer	GTA CGG GCG ACC CCA CGA TGA C	
Probe	ROX – CAC ATC GAT AGA TCA AGG TGC CTA CAA GC – BHQ2	

Primer and probe sequences for MS2 Bacteriophage. The probe was labelled at the 5' end with the fluoresecent reported dye carboxy-X-rhodamine (ROX) and a non-fluorescent quencher, Black Hole Quencher™ 2 (BHQ-2) at the 3' end.

Composition of the second panel of the real-time reverse-transcription polymerase chain reaction assay for the detection of respiratory viruses that included human respiratory syncytial virus, human parainfluenza virus types 1 and 3, and adenovirus.

Residue K represents a G or T residue, residue M represents an A or a C residue, residue R represents an A or G residue, residue W represents an A or T residue, residue Y represents a C or T residue, and residue X is a modified purine base A or G.

Primer or probe	Sequence (5'-3')	Target Gene
HRSV Forward	GGG WGG WGA AGC WGG ATT CTA CC	Nucleoprotein
HRSV Reverse	ACC TCT RTA CTC TCC CAT TAT GCC TAG	
HRSV Probe	6FAM – TAG GCA ATG CWG C C – MGBNFQ	

Primer and probe sequences for human respiratory syncytial virus (HRSV). The TaqMan® MGB™ probe was labelled at the 5' end with the fluorescent reporter dye 6-carboxyfluorescein (6FAM™) and a nonfluorescent quencher (NFQ) at the 3' end.

Primer or probe	Sequence (5'-3')	Target Gene
HPIV1 Forward	GCY CCT TTY ATA TGT ATA CTC AGA GAC CCA	Haemagglutinin
HPIV1 Reverse	TGT TCT TCC AGT TAC ATA YTG TTG CAT AGC	
HPIV3 Forward	GCT CCT TTY ATC TGT ATC CTC AGA GAT CC	
HPIV3 Reverse	TGA TCT TCC CGT CAC ATA CTG TTG CAT G	
HPIV13 Probe	VIC – TGG AGY TAY GCA ATG GG – MGBNFQ	

Primer and probe sequences for human parainfluenza virus (HPIV) types 1 and 3. The combined HPIV 1 and 3 TaqMan® MGB™ probe was labelled at the 5' end with the fluorescent reporter dye VIC® and a nonfluorescent quencher (NFQ) at the 3' end. VIC is a proprietary dye only availble from Applied Biosystems.

Primer or probe	Sequence (5'-3')	Target Gene
AdV Forward	GCC CCA RTG GKC NTA CAT GCA CAT C	Hexon
AdV Reverse	GCC ACX GTG GGR TTY CTR AAC TT	
AdV Probe	ROX – TGC ACC AGA CCC GGR CTC AGR TAC TCC GA – BHQ2	

Primer and probe sequences for adenovirus. The probe was labelled at the 5' end with the fluresecent reported dye carboxy-X-rhodamine (ROX) and a non-fluorescent quencher, Black Hole Quencher™ 2 (BHQ-2) at the 3' end.

Composition of the third panel of the real-time reverse-transcription polymerase chain reaction assay for the detection of respiratory viruses that included human parainfluenza virus types 2 and 4, human metapneumovirus, human rhinovirus, and enterovirus.

Residue R represents an adenine (A) or guanine (G) residue and residue Y represents a cytosine (C) or thymdine (T) residue.

Primer or probe	Sequence (5'-3')	Target Gene
EnV/HRV Forward	CGG CCC CTG AAT GYG GCT AA	3' untranslated region
EnV/HRV Reverse	GAA ACA CGG ACA CCC AAA GTA	
EnV Probe	6-FAM – TCT GYR GCG GAA CCG ACT – MGBNFQ	
HRV Probe	VIC – TCY GGG AYG GGA CCR ACT A – MGBNFQ	

Primer and probe sequences for human rhinovirus (HRV), and enterovirus (EnV). The EnV TaqMan® minor grove binder (MGB™) probe was labelled at the 5' end with the fluoresecent reporter dye 6-carboxyfluorescein (6-FAM) and a non-fluorescent quencher (NFQ) at the 3' end. The HRV TaqMan® MGB™ probe was labelled at the 5' end with the fluorescent reporter dye VIC® and a NFQ at the 3' end. VIC® is a proprietary dye only availble from Applied Biosystems.

Primer or probe	Sequence (5'-3')	Target Gene
HPIV2 Forward	AAG TGY ATG ACT GCT CCT GAT CAR CC	Haemagglutinin
HPIV2 Reverse	TTG CCA ATR TCT CCC ACC ATR GCA TA	
HPIV2 Probe	ROX – TCA GAA TGC CAT CCG CAA GTC AAT GG – BHQ2	
HPIV4 Forward	AAA TGY ATG ACA GCT TAT GAT CAA CCC A	
HPIV4 Reverse	TTT GCA ATR TCT CCC ACC ATR GCA TA	
HPIV4 Probe	ROX – CAG CTG ATA ARG TAG GTG CTT ATA CTA ACA G – BHQ2	

Primer and probe sequences human parainfluenza virus (HPIV) types 2 and 4. Both probes were labelled at the 5' end with the fluorescent reported dye carboxy-X-rhodamine (ROX) and a NFQ, Black Hole Quencher™ 2 (BHQ-2) at the 3' end.

Primer or probe	Sequence (5'-3')	Target Gene
hMPV Forward	CAT CAG GTA AYA TCC CAC AAA AYC AG	Nucleoprotein
hMPV Reverse	GTG AAT ATT AAR GCA CCT ACA CAT AAT AAR A	
hMPV Probe	CY5 – CCY TCA GCA CCA GAC ACA CC – BHQ2	

Primer and probe sequences for human metapneumovirus (HMPV). The probe was labelled at the 5' end with the fluorescent reported dye cyanine (CY)-5 and a NFQ, Black Hole Quencher™ 2 (BHQ2) at the 3' end.

APPENDIX IV: Associations between respiratory pathogens in paediatric patients with respiratory infection

Respiratory Pathogen	No. pathogens	Percentage (%)	Percentage (%) contribution to infection
Negative	13	9.3	9.3
Single bacterial pathogen			
PSEU	1	0.7	9.3
SAUR	7	5.0	
COLI	1	0.7	
CALB	1	0.7	
SPNE	1	0.7	
HINF	1	0.7	
STRB	1	0.7	
Single viral pathogen			
FB	1	0.7	16.4
EnV/HRV	12	8.6	
HRSV subtype A	5	3.6	
HRSV subtype B	2	1.4	
HPIV type 1	1	0.7	
FA type H1	2	1.4	
2 bacterial co-pathogens			
SAUR & PSEU	1	0.7	1.4
HINF & SAUR	1	0.7	
2 viral co-pathogens			
HRSV subtype B & EnV/HRV	1	0.7	1.4
HCoV 229E & EnV/HRV	1	0.7	
2 co-pathogens			
HINF & HPIV type 4	1	0.7	38.6
HINF & HMPV	3	2.1	
COLI & HMPV	1	0.7	
SPNE & HPIV type 3	1	0.7	
HINF & HPIV type 3	2	1.4	
MCAT & HPIV type 3	1	0.7	
HINF & FA type H1	1	0.7	
HINF & FA type H3	1	0.7	
HINF & HRSV subtype B	1	0.7	
SPNE & EnV/HRV	9	6.4	
HINF & EnV/HRV	7	5.0	
MCAT & EnV/HRV	9	6.4	
SAUR & EnV/HRV	3	2.1	
MRSA & EnV/HRV	1	0.7	
PSEU & EnV/HRV	1	0.7	
COLI & EnV/HRV	2	1.4	
MCAT & HRSV subtype A	2	1.4	
SAUR & HRSV subtype A	3	2.1	
SPNE & HRSV subtype A	3	2.1	
SPNE & HRSV subtype B	2	1.4	
3 co-pathogens			
HINF & HRSV subtype B & EnV/HRV	1	0.7	17.1
PSEU & FB & EnV/HRV	1	0.7	
MCAT & HCoV 229E & AdV	1	0.7	
SPNE & HINF & HRSV subtype B	3	2.1	
SPNE & HRSV subtype B & EnV/HRV	2	1.4	
SPNE & MCAT & EnV/HRV	1	0.7	
SPNE & HINF & EnV/HRV	6	4.3	
MCAT & HRSV subtype A & EnV/HRV	1	0.7	
MCAT & HRSV subtype A & HCoV 229E	1	0.7	
SPNE & HRSV subtype A & EnV/HRV	1	0.7	
HINF & HRSV subtype A & EnV/HRV	2	1.4	
SPNE & SAUR & HPIV type 3	1	0.7	
HINF & MCAT & HMPV	2	1.4	
SPNE & HINF & HMPV	1	0.7	
4 co-pathogens			
SPNE & HINF & HMPV & EnV/HRV	1	0.7	3.6
SPNE & HINF & SAUR & EnV/HRV	1	0.7	
SPNE & HINF & EnV/HRV & HMPV	1	0.7	
SPNE & HINF & EnV/HRV & AdV	1	0.7	
HINF & HCoV OC43 & HPIV3 & EnV/HRV	1	0.7	
5 co-pathogens			
SPNE & HINF & HRSV subtype A & EnV/HRV & AdV	1	0.7	2.9
SPNE & HINF & HRSV subtype B & CoV 229E & EnV/HRV	1	0.7	
SAUR & COLI & CALB & HPIV type 1 & ENV/HRV	1	0.7	
SPNE & HINF & SAUR & HPIV type 3 & EnV/HRV	1	0.7	
TOTAL	**140**	**100.0**	**100.0**