Evaluation and adaptation of molecular approaches for detection and characterization of viruses of the respiratory tract

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Submitted by

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University of Westminster
December 2011

A dissertation submitted in partial fulfilment of the requirements of the University of Westminster for the Degree of Doctor of Philosophy

Disclaimer

I hereby declare that this dissertation is the result of my own work conducted between October 2008 and December 2011 under supervision of Dr. Patrick Kimmitt in the School of Life Sciences, University of Westminster, London, UK. This work has not been submitted for any other degree at this, or any other university.

I also declare that this dissertation does not exceed 80,000 words.

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Abstract

This study was designed for the development of novel methods for isothermal amplification of nucleic acids using phi29 and *Bst* DNA polymerases in the detection and characterization of pathogenic viruses of the respiratory tract. High Resolution Melting (HRM) analysis was used in this study for screening similar sequences to avoid unnecessary sequencing from genomic libraries and detection of common pathogenic viruses of the respiratory tract.

Four different structures of DNA linear double stranded DNA (dsDNA, 48 kb), linear single stranded DNA (ssDNA, 3.6 kb), small circular dsDNA (2.6 kb) and a large circular dsDNA (8.6 kb) were used as representatives of different types of viral nucleic acids for the evaluation of phi29-amplification at limiting concentrations. Phi29-amplification of linear ds, large and small circular dsDNA could be amplified 10-100-fold but ssDNA could not be amplified at all. Alternative protocols were adapted for ligating ssDNA and dsDNA to construct large concatemers for efficient phi29-amplification. Phi29-amplification of ssDNA was found to increase >10-fold when it was ligated using T4 RNA ligase in the presence of a large excess of background ssDNA compared to that in the absence of background ssDNA. T4 DNA ligase-mediated phi29-amplification of double stranded cDNA (dscDNA) was found to increase $>10^5$-fold compared to non-ligated dscDNA. CircLigase™ II ssDNA ligase was used for circularization of ssDNA and aid phi29-amplification. CircLigase™ II ssDNA ligase-mediated phi29-amplification of ssDNA was found to increase $>10^7$-fold compared to non-ligated sscDNA.

A method named RT-Bst was developed for simultaneous reverse transcription and *Bst* DNA polymerase amplification of cDNA in the same reaction. In a qualitative PCR assay, RT-Bst was found to be more efficient than the QuantiTect™ reverse transcription kit (QIAGEN, Crawley, UK). The RT-Bst method was used for amplification of cDNA in 70 nasopharyngeal samples for detection of a panel of 12 pathogenic viruses of the respiratory tract. The performance of multiplex RT-Bst PCR detection of pathogenic viruses was comparable to that of multiplex one-step RT-PCR (performed in this study) and more efficient than immunofluorescence (performed by a hospital diagnostic laboratory). The RT-Bst protocol was further modified using tailed random primers to develop another protocol for whole genome amplification designated RT-Bst Single Primer Amplification (RT-Bst SPA) as a cheaper alternative to sequence independent single primer amplification.

HRM analysis was used in this study to demonstrate its application for low cost screening of similar sequences to avoid unnecessary sequencing task from whole genome libraries prepared from MS2 RNA and tissue culture media positive for influenza B and hRSV. HRM analysis was also used for rapid and low cost detection of common pathogenic viruses of the respiratory tract.

It is proposed that RT-Bst and RT-Bst SPA have the potential for sequence independent amplification of RNA sequences for subsequent multiplex PCR detection, and other downstream applications. HRM analysis can be used for rapid and cost effective detection of pathogenic viruses of the respiratory tract. However, a more detailed study will be required for further optimization and validation of the developed protocols for appropriate commercialization as kits.

Dedicated to

My parents

Acknowledgements

I would like to thank my director of studies Dr. Patrick Kimmitt for his continuous support and spontaneous inspiration throughout the study. I am very grateful to my second supervisor Dr. Mark O. Clements for his valuable comments, suggestions, support with his knowledge and guidance. I would also like to thank Dr. Pamella Greenwell for her voluntary advice and critical review of my research work.

I am thankful to Dr. Mark Atkins, Chelsea and Westminster Hospital for his cooperation for obtaining nasopharyngeal samples necessary for this study. Thanks to Jeremy Bishop, St. Mary's Hospital, Imperial College London, UK for providing tissue culture positive virus samples for this study. Special thanks to Professor Anne Moir, University of Sheffield for providing bacteriophage lambda particles for this study.

I also express my gratitude to other members of the laboratory C5.11 in no particular order, Aaliya, Emma, Karen, Karima for their cooperation and moral support. I am also thankful to the technical staff for their support and cooperation.

This study was supported by the Cavendish Scholarship of the University of Westminster. I am also thankful to the Charles Wallace Bangladesh Trust for their support towards this study.

I am extremely indebted to my wife and beautiful daughter for their relentless support during strive throughout my study. My wife and daughter were beside me all the time of my hardship and cheerfulness during my study. My special thanks to my parents, brother and sisters for their inspiration, moral and financial supports for my doctoral studies.

I am immensely grateful to Almighty Allah for making me so fortunate to avail such a wonderful opportunity to pursue PhD in one of the reputed universities in the world.

Thanks to all!

Md. Shahidul Kabir
London
December 2011

Table of Contents

Abbreviations	
A	adenine
bp	base pair
BSA	bovine serum albumin
C	cytosine
cDNA	complementary deoxyribonucleic acid
C_T	cycle threshold
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dscDNA	double stranded complementary deoxyribonucleic acid
cdsDNA	circularized double stranded deoxyribonucleic acid
cdscDNA	circularized double stranded complementary deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
g	gram
G	guanine
gDNA	genomic deoxyribonucleic acid
GE	genome equivalent
h	hour
hRSV	human respiratory syncytial virus
hRV	human rhinovirus
IDT	integrated DNA technology
K	kanamycin
kb	kilo base
kDa	kilo Daltons

λ	lambda DNA
l	litre
LB	Luria Bertani
M	molar
m	metre
MDA	multiple displacement amplification
µ	micro
min	minute
mRNA	messenger ribonucleic acid
NASBA	nucleic acid sequence based amplification
NGS	next generation sequencing
ng	nano gram
NHS	national health services
nt	nucleotide
PCR	polymerase chain reaction
pg	picogram
pfu	plaque forming unit
PIV	parainfluenza virus
RACE	Rapid amplification of cDNA ends
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SISPA	sequence independent single primer amplification
snRNA	small non-coding ribonucleic acid

SDW	sterile distilled water
sscDNA	single stranded complementary deoxyribonucleic acid
T	thymine
TAE	Tris-acetate EDTA
tRNA	transfer ribonucleic acid
T_m	melting temperature
U	uracil
UTR	un-translated region
UV	ultra-violet
VDISCA	virus discovery cDNA AFLP
WGA	whole genome amplification

Chapter 1

Introduction

1.1 Respiratory tract infection

The development of molecular methods has revolutionised not only the detection of unknown viruses but also the detection of divergent novel viruses of the respiratory tract. A number of previously unknown pathogenic viruses of the respiratory tract for example, human metapneumovirus (hMPV) (van den Hoogen *et al*., 2001), human coronavirus (hCoV) NL63 (van der Hoek *et al*., 2004) and HKU1 (Woo *et al*., 2005), human bocavirus (hBoV) (Allander *et al*., 2005) have been reported in the last two decades. Human viral infections may affect all age groups with a range of severity of infections which may depend on different environmental factors and clinical conditions (Weber, 2009). Respiratory tract infections can be caused by a variety of microorganisms but the majority of such infections are caused by viruses (Makela, *et al*., 1998). Some of these viruses such as influenza A may cause severe disease and can lead to pandemic spread with high morbidity and mortality (Grasselli *et al*., 2011). Other viruses such as bocavirus may cause less severe infection leading to common cold symptoms (Lindner *et al*., 2008). The patterns of respiratory tract infections in common cold are complex due to the potential incidence of multiple viruses in respiratory tract infections in humans.

Upper respiratory tract infections (URTI) can be caused by a wide variety of viruses, including rhinovirus, coronavirus, influenza A and B, parainfluenza, respiratory syncytial virus, adenovirus, metapneumovirus, and enterovirus; as well as by bacteria, including *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Bordetella pertussis*, and *Haemophilus influenzae* (Costa *et al.,* 2006; Louie *et al.,* 2005; Makela *et al.,* 1998). The clinical symptoms of URTI are highly variable and cannot be used to identify the aetiological agents. Traditional methods for the identification of URTI pathogens, including culture and serology are effective for many viral pathogens but are laborious and time consuming. Serological tests, for example immunofluorescence, can be rapid but are often not sensitive or specific enough to detect all varieties of pathogenic viruses. In one study enzyme immunoassays

(EIA) and direct immunofluorescence (DIF) assays showed sensitivities of 85% and 60-80%, respectively for detection of influenza virus (Cram *et al.,* 1999). Although viral culture is generally accepted as the gold standard for diagnosis, PCR can be more sensitive (Templeton *et al.,* 2004). In one study, culture detected 40% of rhinovirus infections in patients while PCR detected 51.5% (Makela *et al.,* 1998). Similarly, an influenza virus surveillance study showed that 18% of samples were positive by culture and 28% were positive by TaqMan-PCR (Schwieger *et al.,* 2000). However, TaqMan-PCR is relatively expensive and is not regularly used for routine laboratory diagnosis of pathogenic viruses in developing countries.

Respiratory tract viral infection can be self-limiting upper respiratory tract infections or more serious forms of lower respiratory tract infections. Rapid and accurate detection of pathogenic viruses of the respiratory tract can help the early diagnosis of pathogenic viral infection, decrease the duration of hospitalization and reduce management costs, as well as avoiding additional laboratory testing and unnecessary administration of antibiotics (Barenfanger *et al*., 2000; Woo *et al*., 1997).

1.2 Global picture of respiratory tract viral infections

Respiratory tract infection is a major cause of disease burden compared to other causes of infection (Mizgerd, 2006). Respiratory tract infection is one of the leading causes of disease and resulting in mortality rate of 18% in children less than 5 years old in 2002. The next most common infections are diarrhoeal diseases (15%) and malaria (11%) around the world (WHO, 2005). Morbidity due to respiratory tract infection in this age group varies in different parts of the world. It was reported that 22% and 26.7% of all hospitalized patients had respiratory illness in the UK (Nicholson *et al*., 2006) and Belgium (Massin *et al*., 2006), respectively. Amongst all other viral infections in humans, influenza was found to be associated with approximately 250,000–500,000 deaths globally each year (World Health Organization, 2009). The actual mechanism for spread of influenza

seasonally worldwide is not clearly understood but is found to be associated with a number of factors including environmental conditions and patient health (Cannell *et al*., 2006; Dowell, 2001; Lipsitch and Viboud, 2009; Lofgren *et al*., 2007). Influenza epidemics occur in the northern and southern hemispheres during their respective winters (Finkelman *et al*., 2007; Hope-Simpson, 1981) while increased influenza activity is found to be linked to the rainy season in several tropical populations (Chew *et al*., 1998; Chumkiew *et al*., 2007; de Mello *et al*., 2009; Dosseh *et al*., 2000; Moura *et al*., 2009; Rao and Banerjee, 1993). Other factors linked to influenza epidemics include cold temperatures (Davey and Reid, 1972), low indoor humidity (Hemmes *et al*., 1962), and minimal solar radiation (Hope-Simpson, 1981). However, a detailed study needs to be done for comprehensive description of the global pattern of seasonal activity of influenza.

1.3 Common pathogenic viruses associated with respiratory tract infections

Respiratory tract infections are commonly associated with a range of pathogenic viruses and clinical manifestations. There are approximately 200 known respiratory tract viruses of the *Adenoviridae*, *Parvoviridae*, *Orthomyxoviridae*, *Paramyxoviridae*, *Picornaviridae* and *Coronaviridae* families associated with respiratory syndromes (Abed and Boivin, 2006). Only the common pathogenic viruses associated with respiratory tract infections are described in the following section.

1.3.1 Influenza virus

Human influenza viruses are enveloped, single-stranded, negative-strand, segmented RNA viruses that belong to the family *Orthomyxoviridae*. Influenza virus infections may lead to febrile respiratory illnesses and annual epidemics prevailing for about 3 to 8 weeks (Atmar, 2007; Harman, 1999). Influenza infection can spread globally in the pandemic form through the spread of novel virus types. These novel types of influenza viruses emerge following genetic reassortment of

the haemagglutinin (HA) and neuraminidase (NA) genes (Stockman *et al*., 2006). Although three subtypes: H1N1, H2N2 and H3N2 were found to be associated with seasonal variations in the last 100 years, a new variant of H1N1 transferred from swine to humans was responsible for a pandemic (worldwide) outbreak in 2009 (Al Johani *et al*., 2011). In addition, the transfer of subtype H5N1 from birds to humans triggered the severe outbreak in Southeast Asia in 2003 and caused significant mortality and morbidity in humans (Adams and Sandrock, 2010). Although clinicians sometimes use laboratory diagnosis, they often diagnose patients solely based on the presence of influenza-like symptoms. Identification of influenza virus in a hospital environment is important for appropriate control of the spread of infection for example by droplet isolation. The spread of influenza infection in a hospital can be disastrous for immunocompromised patients for example, transplant recipients or cancer patients, and can cause serious complications leading to death. Transplant recipients may acquire viruses through the donor-derived infections, reactivation of endogenous latent viruses or from the community. Due to the immunocompromised states of these patients they are susceptible to infections from any opportunistic pathogens. Administration of specific antiviral agents for example, M2 channel inhibitors (amantadine and rimantidine) can be effective in controlling the spread of infections between patients. However, for effective treatment of patients M2 channel inhibitors need to be given within first 24 hours of infection. Therefore, a rapid and accurate laboratory diagnosis of influenza virus is of prime importance.

Avian influenza virus (H5N1) was first detected in Hong Kong in 1997 (Gillim-Ross, 2006) and resulted in death for 6 of 18 reported cases. A large number (1.5 million) of infected chickens were culled in Hong Kong to limit the spread of the virus from the infected chickens. Millions of wild and domestic birds were infected by H5N1 in Southeast Asia in 2003. Subsequently, 229 people died out of 359 people who became infected in 14 countries in 2008 (WHO, 2008).

1.3.2 Human parainfluenzavirus (PIV)

Human parainfluenzaviruses are enveloped single-stranded negative-sense RNA viruses grouped within the family of *Paramyxoviridae*. There are four distinct serotypes of PIV associated with human infections (Beck and Henrickson, 2010; Henrickson, 2003). The mode of transmission and pathogenesis of PIVs are similar to those of influenza viruses. PIV1 and PIV2 are found to be the major cause of croup in children (Wright, 2005; Denny *et al*., 1983) and upper respiratory tract infection (URTI) in all other age groups (Mahony, 2008; Henrickson, 2003). PIV3 is more likely to be associated with severe lower respiratory tract infection (LRTI) in children. Infections caused by the PIV4 (Subtypes PIVa and PIVb) are less prevalent and less studied compared to the other common viruses of the respiratory tract (Beck and Henrickson, 2010).

1.3.3 Human respiratory syncytial virus (hRSV)

Human respiratory syncytial virus (hRSV) is a member of *Paramyxoviridae* and contains a single-stranded negative-sense RNA genome. RSV has been reported to be the major cause of bronchiolitis and pneumonia in children under the age of 2 years (American Academy of Pediatrics, 2003). There are two subtypes (A and B) of hRSV found to be associated with infections caused by hRSV. There were over 100,000 hospitalization and 4,500 deaths associated with hRSV infection each year in the USA, with an estimated cost to the economy of $300 million (Hall *et al*., 2000).

1.3.5 Adenovirus

Adenoviruses are double-stranded DNA viruses of the *Adenoviridae* family and have more than 51 known serotypes which are categorized into six subgenera A to F. Adenoviruses can also cause other types of infections for example, conjunctivitis, keratoconjunctivitis, and acute gastroenteritis. Amongst the 51 known serotypes the ones associated with the respiratory tract infections include 1 to 5, 7, 14, 19 and 37. Adenoviruses generally spread through inhalation of droplet nuclei and infect children under the age of 10 years old (Fox *et al*., 1969). The

incidence of adenovirus infections in haematopoietic stem cell transplant patients have been reported by different workers which range between 4.9% and 20.9% (Blanke *et al*., 1995; Wasserman *et al*., 1988; Shields *et al*., 1985; Munoz *et al*., 1998).

1.3.6 Rhinovirus (RV)

Rhinoviruses are single-stranded positive-sense RNA viruses of the *Picornaviridae* family of which more than 200 serotypes have been identified. Rhinoviruses cause approximately two-third of cases of common cold in humans and have been found associated with asthma exacerbations in chronic lung disease (Kling *et al*., 2005; Gern, 2002; Douglas, 1970). Rhinoviruses and enteroviruses belong to the same family *Picornaviridae* and often detected using primers specific for the highly conserved 5'-noncoding region (NCR). However, designing a second set of primers targeting the VP1 or VP4 genes can be useful to differentiate between rhinoviruses and enteroviruses (Kiang *et al*., 2007). RT-PCR, nucleic acid sequence based amplification (NASBA) have been found to be more sensitive than conventional culture techniques for the detection of rhinoviruses (Blomqvist *et al*., 1999; Halonen *et al*., 1995; Hyypia¨ *et al*., 1998; Loens *et al*., 2006; Steininger *et al*., 2001; Vuorinen *et al*., 2003).

1.3.7 Enterovirus and Parechovirus

Enterovirus and Parechovirus are single-stranded positive-sense RNA viruses of the *Picornaviridae* family. The genus *Enterovirus* and *Parechovirus* have been shown to have 63 and 4 distinct members, respectively. Enteroviruses are prevalent worldwide infecting an estimated 1 billion people each year. These infections are common in infants and young children especially under the age of 1 year (Benschop *et al*., 2006a and 2006b; Boivin *et al.*, 2005). Enterovirus infections can cause a range of disease conditions starting from mild respiratory symptoms (Cherry, 2004) to serious forms such as poliomyelitis and aseptic meningitis. Although parechoviruses mainly cause respiratory tract and gastrointestinal infections (Benschop *et al*., 2006b; Kuypers *et al*., 2006) human

parechoviruses can cause acute flaccid paralysis and severe encephalitis (Figueroa *et al*., 1989; Koskiniemi *et al.*, 1989).

1.3.8 Human metapneumovirus

Human metapneumoviruses consist of two major groups (A and B) and four subgroups. Human metapneumovirus is an RNA virus of the *Paramyxoviridae* family which were discovered in 2001 (van den Hoogen *et al.*, 2001). It can cause mild to severe respiratory tract infections with symptoms very similar to those caused by hRSV and can lead to the development of bronchiolitis and pneumonia (Konig *et al*., 2004; Van den Hoogen *et al*., 2003). Human metapneumovirus (hMPV) can cause sporadic infection throughout the year (Williams *et al*., 2004) with predominant outbreaks in the winter and spring months of temperate climates (Kaida *et al*., 2006; Robinson *et al*., 2005; Van den Hoogen *et al*., 2004). This virus can spread through aerosols, droplets or contaminated surfaces and infect people of all ages primarily infants (Tang *et al*., 2007; von Linstow *et al*., 2006).

1.3.9 Coronavirus (CoV)

Coronaviruses are single-stranded positive-sense RNA viruses belonging to the *Coronaviridae* family. Of the five types of coronaviruses reported so far, HCoV-229E and NL-63 belong to group I whereas OC43, SARS-CoV and HKU1 belong to the group II CoVs. CoV OC43 and 229E have been found to be associated with respiratory tract infection in immunocompromised children and hospitalized elderly patients (Falsey *et al*., 2002; Pene *et al*., 2003). The worldwide outbreak of severe acute respiratory syndrome (SARS) from 2002 to 2003 was associated with SARS-CoV of group II CoV.

1.3.10 Human bocavirus (hBoV)

Human bocavirus is a single-stranded DNA virus of the *Parvoviridae* family. Human bocavirus was discovered by Allander *et al*. (2005) using a large-scale molecular viral screening and DNase Sequence Independent Single Primer Amplification (SISPA) technique. Based on several studies it was found that about

2% to 19% of respiratory tract infections were caused by hBoV. Bocavirus infections are detected mainly by molecular techniques (Lu *et al*., 2006; Neske *et al*., 2007; Choi *et al*., 2008) as it is not yet possible to grow isolates in cell culture.

1.3.11 Parvovirus Type 4 and 5 and Mimivirus

Parvovirus type 4 and 5 were found in the serum or plasma samples of 70% of HIV infected patients (Manning *et al*., 2007). Parvoviruses were also found in 4% of manufactured plasma and in healthy blood donors (Fryer *et al*., 2007). These types of viruses are considered as potential pathogens of the respiratory tract as are two other parvoviruses, parvovirus B19 and HBoV that are associated with respiratory tract infections.

Mimivirus is a large DNA virus considered as a potential pathogen of the respiratory tract. Antibodies to mimivirus could be detected in 9.7% of community-acquired pneumonia cases compared to 2.3% of healthy controls (La Scola, *et al.*, 2005). Mimivirus DNA was also found in bronchoalveolar lavage specimens from intensive care unit patients (La Scola, *et al.*, 2005). However, in another study mimivirus sequence could not be detected by real-time PCR in 496 pneumonia patients (Dare *et al*., 2007). Further research is necessary in order to establish the true pathogenicity of these viruses.

1.4 Traditional diagnostic methods for viruses of the respiratory tract

Serology has been used as the major diagnostic approach during the last two decades for the detection of respiratory tract pathogenic viruses. The most common serological tests used for diagnosis of respiratory tract viral infections are the haemagglutination inhibition (HAI) test, complement fixation and enzyme immunoassay (EIA). HAI was used for determining the subtyping H1 and H3 of influenza and influenza A virus infections. Detection of cytopathic effect (CPE) by haemagglutinating viruses may be improved by haemadsorption with red blood cells. The efficiency of cell culture was also improved in the early 1990s with the

introduction of shell vial culture (SVC) with specific monoclonal antibodies. This approach reduced the cell culture time from 8-10 days to 1-2 days. Direct fluorescent antibody (DFA) staining of cells from nasopharyngeal swabs is another technique commonly used in many laboratories for rapid detection of viruses and takes around 3 hours to complete. Enzyme immunoassays (EIAs) were introduced in the 1980s and 1990s but were found to be less sensitive than molecular methods. Consequently, more sensitive molecular techniques such as PCR, nucleic acid sequence based amplification (NASBA), loop-mediated isothermal amplification (LAMP) were developed as more sensitive detection methods for pathogenic viruses of the respiratory tract.

1.5 Molecular methods for detection of pathogenic viruses of the respiratory tract

There are a number of molecular methods developed in the last two decades for the detection of pathogenic viruses of the respiratory tract. Only the most relevant methods have been described in the following sections.

1.5.1 Important aspects of molecular detection of pathogenic viruses of the respiratory tract

Interpretation of the results of molecular detection of pathogenic viruses of the respiratory tract is critical for accurate diagnosis of infections of the respiratory tract. Several important factors need to be considered for interpretation of the detection result such as, viral shedding, quantitation of virus concentration which is described in the following sections.

1.5.1.1 Viral infection and shedding

Collection of patient samples during the early stage of infection is a prerequisite for reliable diagnosis of the respiratory tract infection (Antonishyn and Levett, 2010). The incubation period, infection period and mode of transmission of respiratory tract viruses can vary for different viruses. In a recent multiplex-real-time detection

study of fifteen respiratory tract viruses demonstrated that the detection of viruses is associated with the duration of symptoms (Brittain-Long *et al*., 2009). Detection rate of viruses was higher (51%) in patients having symptoms of six days or less and compared to 30%, $p<0.01$ for patients having symptoms of seven days or more. A similar decline in the detection of RSV has been reported by other workers (Gerna *et al*., 2008; Campanini *et al*., 2007). In another study using real-time PCR it was found that the threshold cycle (C_T) values increased with the duration of symptoms in case of the enveloped respiratory tract viruses (Brittain-Long *et al*., 2010). Threshold cycle value is the cycle number at which fluorescence generated within a reaction crosses the background fluorescence. Background fluorescence is determined from the fluorescence produced by a no template control. The C_T value is inversely proportional to the template concentrations in the quantitative real-time PCR reaction. These real-time PCR findings suggest that the concentrations of viruses decrease gradually after the onset of infection (Brittain-Long *et al*., 2010). In the same study it was found that concentration hRV declined slowly and could still be detected after 10 days of infection. In other studies hRV and hEV were found to shed from respiratory mucous for several weeks and bocaviruses were found to shed for several months (Von *et al*., 2008).

1.5.1.2 Significance of a positive result in a molecular method

Molecular diagnostic tests are highly sensitive compared to older techniques, such as culture and immunofluorescence (Antonishyn and Levett, 2010; Beck and Henrickson, 2010). A positive result may reflect a carrier state other than active infection state. In a previous study Creer *et al*., (2006) found that 2% and 6% of the healthy controls were positive for hRV and influenza A virus, respectively. The presence of hRV in healthy children is also associated with asymptomatic carriage or prolonged shedding of the virus (Nokso-Koivisto *et al*., 2002; Winther, *et al*., 2006; Van Der Zalm, *et al*., 2009; Herberhold, *et al*., 2009). In another study hRV could be detected by PCR up to two weeks after infection or more than two weeks after appearance of symptoms (Brittain-Long *et al*., 2010). Immunocompromised

patients may not show any clinical symptoms but shed a detectable amount of respiratory tract viruses, for example, RSV (Ison, 2007; Peck *et al*., 2007). Some other viruses such as human bocavirus can be secreted for several months (Von Linstow *et al*., 2008; Martin *et al*., 2010; Don *et al*., 2011). Quantification of pathogenic viruses in clinical specimen may help to determine the true causative agents by determining and comparing the threshold cycle (C_T) values of different agents (Utokaparch *et al*., 2011; Gerna *et al*., 2009; Brittain-Long *et al*., 2010). Use of a panel of viruses can be more helpful for detection of multiple infections or for reconfirmation of a negative result. In such studies Influenza A and hRSV are strongly associated with clinical symptoms compared to other viruses thereby detection of low level of these viruses may be of clinical relevance. However, more clinical studies are required to standardize a quantitative molecular method to define cut off level for different clinical situations. Quantitative molecular methods will be more useful if clinical specimens are collected from nasopharynx and/or oropharynx in the early stage of infection containing high titre of viruses (Brittain-Long *et al*., 2008).

1.5.1.3 Unbiased approach for detection of a panel of viruses

Respiratory tract viral infection is associated with a number of pathogenic viruses. There are approximately 200 known viruses that have been reported to be associated with respiratory tract infections (Abed and Boivin, 2006). A number of new virus types have been reported since 2000 for example, avian influenza viruses (H5N1, H7N7 and H7N3), human metapneumovirus (hMPV), severe acute respiratory syndrome (SARS) CoV and human CoVs (hCoV) NL63 and HKU1 (reviewed by Mahony, 2008). Respiratory tract infections can cause mild infection such as, self-limiting upper respiratory tract infection to more serious lower respiratory tract infections. Conventional methods such as culture and immunofluorescence are still used in diagnostic laboratories for detection of respiratory tract viruses but they are often laborious, time consuming and less sensitive (Tregoning and Schwarze, 2010). With the development of multiplex molecular tests, it is now possible to detect 15-20 different viral agents

simultaneously. This increased capacity not only improves the rate of virus detection but has also expanded our knowledge of the aetiology of respiratory tract infections. For example, Human RV and human CoV were previously recognised as mild causes of upper respiratory tract infections but have now been found to be associated with severe infections of lower respiratory tract (reviewed by Mahony, 2008; Renwick *et al*., 2007; Louie *et al*., 2009). However, most of the respiratory tract viruses can show similar clinical manifestations thereby it is very difficult to diagnose any infection based on their clinical manifestations (Gravenstein *et al*., 2000; Li *et al*., 2007; Mahony, 2007c). It has been reported that multiple respiratory tract viral infections could be detected in 10% of the respiratory specimens (reviewed by Mahony, 2008). Moreover, multiple viral infections were found to be associated with severe clinical symptoms (Utokaparch *et al*., 2011; Harvala *et al*., 2008; Schildgen *et al.*, 2008; Paranos-Baccala *et al*., 2008). The inclusion of a panel of respiratory tract pathogenic viruses will not only help to diagnose infections more accurately but also reduce the cost associated with the individual test.

1.5.2 PCR and RT-PCR

PCR is a very sensitive technique for detection of any viral DNA genome target (Saiki *et al*., 1988). Primer design is very important in amplification and detection of a specific or multiple targets. In virology research primers are generally designed for detection of the relatively conserved sequences for example, replication genes and matrix protein coding genes for detection of different members of the same species or family. For example primers designed for detection of the hexone gene of adenovirus can detect 18 genotypes in urine samples (Echevarria *et al.*, 1998b). Multiple sets of primers specific for different viruses are used for the detection of multiple viruses in multiplex PCR assay. In order to amplify all of the targets in the same amplification reaction it is necessary to optimise the thermocycling conditions for example, annealing temperature and extension time for all of the primers. DNA amplified by PCR can be detected

through separation of the DNA on agarose gel and detection of the appropriate band or by using sequence specific oligonucleotide probes (Osiowy, 1998).

Both one-step and two-step RT-PCR methods are used for detection of RNA viruses in clinical samples. In two-step RT-PCR, cDNA is first prepared from the viral RNA which is then used as template for further detection of target sequences by PCR. In one-step RT-PCR viruses can be detected directly from extracted RNA. One-step RT-PCR is more sensitive than the two-step RT-PCR because it includes relatively larger amount of RNA for reverse transcription and PCR amplification. One-step RT-PCR also amplifies cDNA using PCR in the same reaction after reverse transcription of RNA which excludes the possibility of pipetting errors and carryover contaminations. One-step RT-PCR was used to successfully amplify the matrix, nucleoprotein (NP) and haemagglutinin (HA) genes of influenza A and B viruses (Poddar, 2002). Amplified PCR products can be separated on agarose gels for detection of appropriate sizes of the bands. Multiplex RT-PCR can be developed after optimizing different primers for the detection of a panel of pathogenic viruses (Sanghavi *et al*., 2011; Bellau-Pujol *et al*., 2005). Multiplex RT-PCR has also been used for simultaneous detection of influenza A and differentiation of avian H5, H7 and H9 subtypes (Xie *et al*., 2006).

1.5.3 Quantitation of virus using molecular methods

Molecular methods are more sensitive than conventional methods and also allow quantification of the viral load. Detection of viral load can be useful for diagnosis of infection, to determine the shedding of viruses in different stages of infection and for monitoring of therapy. Rapid and accurate quantification of viral load by real-time quantitative PCR has been found to be useful in clinical settings for detection and quantitation of 13 common respiratory tract viruses using fluorescence resonance energy transfer (FRET) hybridisation probes (Lassauniere *et al*., 2010; Kaltenboeck and Wang, 2005). Special clinical management policies and infection control measures (To *et al*., 2010) should be taken if high viral load and prolonged viral shedding is found in young children. It was also possible to determine the

concentrations of different types of adenoviruses using multiplex type-specific real-time PCR (Boivin *et al*., 2003). Recently, a super high speed real-time quantitative PCR was introduced by Sakurai *et al*. (2011) for rapid and sensitive detection of influenza viruses in less than 20 minutes.

1.5.4 Loop-mediated isothermal amplification (LAMP) and reverse transcription loop-mediated isothermal amplification (RT-LAMP)

LAMP is a very sensitive and rapid isothermal amplification method used for the detection of DNA. The principle of LAMP is based on autocycling strand-displacement synthesis of DNA (Notomi *et al*., 2000). Two inner and two outer primers are used with *Bst* DNA polymerase for isothermal amplification of a target sequence in such amplification. *Bst* DNA polymerase is the large fragment of DNA polymerase derived from *Bacillus stearothermophilus* which has 5'→3' polymerase activity but no 5'→3' exonuclease activity (Aliotta *et al*., 1996). As a result of the LAMP reaction a mixture of stem-loop DNAs of different stem lengths comprising of multiple loops are produced. A positive amplification can be easily monitored either by gel electrophoresis or measuring the concentration of magnesium pyrophosphate (Mori *et al*., 2004). LAMP has been applied successfully for detection of RNA sequences by adding an initial reverse transcription (RT) step and additional pair of primers called RT-LAMP (Parida *et al*., 2004; Yoda *et al*., 2007). LAMP was found to be 100 times more sensitive than PCR for detection of tomato yellow leaf curl virus DNA (Qu *et al*., 2010). RT-LAMP was also found to be 10 times more sensitive than RT-PCR for detection of Taura syndrome virus (TSV) RNA (Kiatpathomchai *et al*., 2007).

1.5.5 Nucleic acid sequence-based amplification (NASBA)

In this technique an RNA template is amplified through simultaneous activities of three enzymes namely reverse transcriptase, RNase H and DNA-dependent RNA polymerase (Guatelli *et al*., 1990) (Figure 1.1). Initially a DNA primer hybridizes to the T7 promoter and is extended by reverse transcriptase (RT). RNase H degrades the RNA from the cDNA/RNA hybrid and double-stranded DNA is

synthesized by reverse transcriptase (RT) using a second primer. Multiple copies of RNA are formed from this DNA template by T7 polymerase. Amplified RNA can be detected by electrochemiluminiscence and RT-PCR and has been used for rapid detection of various viruses including West Nile virus and St. Louis encephalitis virus (Lanciotti and Kerst, 2001).

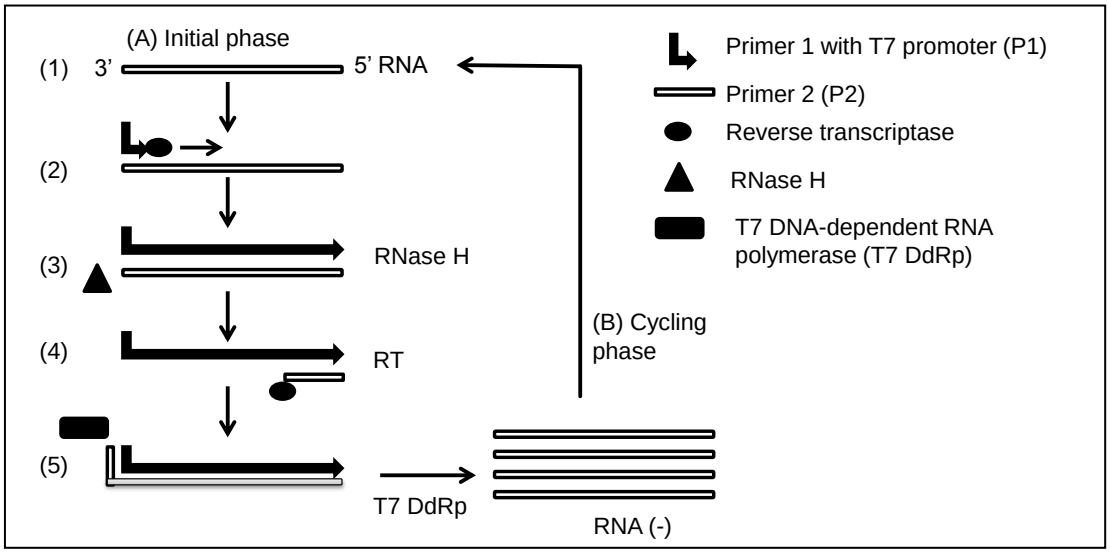

Figure 1.1: Nucleic acid sequence based amplification (NASBA). (A) Initial phase: (1) template RNA; (2) reverse transcription with primer (P1) containing T7 promoter; (3) RNase H degrades RNA from cDNA-RNA hybrid; (4) Primer 2 (P2) binds to the promoter sequence and complementary DNA strand is extended by reverse transcriptase (DNA dependent DNA polymerase activity); (5) double stranded T7 promoter acts as a self-sustained template for T7 DNA-dependent RNA polymerase to generate complementary RNA; (B) Cycling phase: each RNA can be copied in cyclic order and amplified exponentially. Figure adapted from Asiello and Baeumner, 2011.

1.5.6 Nucleic acid hybridization method

The nucleic acid hybridisation technique can be used for the detection of specific nucleic acid sequences for example; viral genomes. A short labelled probe of 20-30 bases is hybridized to a unique complementary piece of viral genome. This

short probe can be either DNA or RNA (riboprobe). This technique relies on the signal amplification system rather than amplification of the target genome. Signals from the hybridized probes can be amplified through chemiluminiscence or a hybrid-capture assay for quantitation of viruses. The nucleic acid hybridisation method has been used to monitor human immunodeficiency virus type 1 (HIV-1) RNA in plasma samples from patients enrolled in clinical trials for antiretroviral and immune-based therapies (Dewar *et al*., 1994).

1.5.7 Microarray analysis of virus sequences

Microarray technology is based on nucleic acids hybridisation method used for the detection and characterisation of different sequences. A range of single-stranded DNA oligonucleotide probes are spotted on a small glass slide, membrane or coated quartz microchip surface for hybridization and detection of complementary nucleic acid sequence. Genomic DNA or RNA is extracted from a desired sample and the target sequences are amplified and labelled with fluorescent markers. These labelled sequences are allowed to hybridize with complementary sequences in the array. Subsequently, fluorescence is monitored to determine the presence of particular sequence. Microarrays have already been used for the detection and typing of pathogenic viruses from the respiratory tract (Mehlmann *et al*., 2007; Thompson *et al*., 2003; Mahony *et al*., 2007a and 2007b). A commercial microarray device CLART® PneumoVir (Genomica, Coslada, Madrid, Spain) was developed for the detection of multiple respiratory tract viral pathogens and is also considered to be cheaper than multiple multiplexed conventional PCR-based assays (Frobert *et al*., 2011). There is an enormous prospect for adopting microarray platforms for cheaper detection of multiple infectious agents such as bacteria, virus and fungi in patient samples. Microarray analysis can also be designed for detection and characterisation of different genotypes such as drug resistant types.

1.5.8 Multiplex PCR and emerging technologies for the detection of respiratory tract pathogens

The application of molecular techniques is becoming widespread due to their performance, rapid turnaround time and ability to detect a range of different pathogens compared to other conventional techniques. It is also more convenient to set up multiplex assays after multiplex PCR amplification of the metagenome using modern techniques for example, ResPlex™ technology and Infinity™ system which are described in the following sections. Several emerging techniques are described in this section but none of them have yet been approved by the US Food and Drug Administration (FDA) with the exception of the ProFlu-1™ assay.

1.5.8.1 Micro-bead suspension array (EraGen Biosciences) multiplex PCR

In this technique multiple targets are amplified using multiplex PCR. Consequently, amplified products are hybridized to target-specific capture probes covalently bound to colour-coded beads. Hybridization of coloured beads is detected using a dual-laser detection device for determining the presence or absence of particular pathogens in the sample. This technology has been successfully applied for the detection of common pathogenic respiratory tract viruses (Mahony *et al*., 2007a) and typing of human papillomaviruses (HPV) (Schmitt *et al*., 2006). This technology offers detection of 100 different types of sequences in the same reaction.

1.5.8.2 ResPlex™ Technology (Qiagen)

This technology involves multiplex PCR amplification followed by bead-based detection such as Luminex xMAP. This technology is suitable for detection of ≥15 pathogens from a single test. ResPlex™ I and ResPlex™ II are designed for detection of bacterial and viral respiratory pathogens, respectively. The ResPlex™ system was used for detection of 21 common pathogens including bacteria and viruses from patients having acute respiratory infections (Brunstein *et al*., 2008).

ResPlex™ test was found to have a sensitivity of 84%-100% compared to direct fluorescent antibody (DFA) test for detection of influenza A and B, parainfluenza 1-3 and RSV. However, ResPlex™ was found to be less than 10% sensitive compared to DFA when tested for adenovirus.

1.5.8.3 Infiniti™ System

In the Infiniti™ system (AutoGenomics) target sequences are amplified initially using multiplex PCR for subsequent detection using automated microarray hybridization in Infiniti™ analyser. Unincorporated nucleotides are removed through enzymatic digestion and amplicons are labelled using fluorescent nucleotides. In a recent study, this microarray approach was compared with quantitative real-time PCR for detection of 23 common respiratory tract viruses in children ≤3 years of age (Raymond *et al*., 2009). Microarray analysis was found to be ≥90% sensitive for all viruses other than adenoviruses and coronavirus NL63. Although throughput was higher for microarray analysis for detection of >15 targets, a singleplex real-time PCR was found to be more sensitive for detection of an individual virus (Raymond *et al*., 2009).

1.5.8.4 Jaguar system

The Jaguar system (BD Diagnostic) is an automated nucleic acid extraction and real-time PCR detection technology for identification of multiple respiratory pathogens. Nucleic acid extraction occurs in individual cartridges. The amplification cartridge can accommodate 12 reactions of 4 µl each with multiplexing capabilities of 2-6 targets. It was found that the sensitivities for the detection of influenza A, influenza B and RSV were 100%, 90% and 100%, respectively compared to tissue culture detection (Beck *et al*., 2010).

1.5.8.5 FilmArray™ technology

The FilmArray™ system (Idaho Technologies) is an integrated automated system for nucleic acid extraction, nested PCR, detection and data analysis for the

diagnosis of numerous viral and bacterial respiratory pathogens in a single-use pouch. A PCR reaction is performed in two steps; the first step is a multiplex PCR containing primers for all of the target sequences and the second step targets a specific pathogen. This technique has the potential for the detection of >15 target sequences in the same reaction within an hour (Rand *et al*., 2011; reviewed by Caliendo, 2011).

1.5.8.6 Scalable target analysis routine (STAR) technology

STAR technology (PrimeraDx) is a blend of PCR with a capillary electrophoresis system. Different targets are identified based on the sizes of the amplified products. It is possible to detect large number of pathogens (>20) in a single reaction. This system is still under study for the detection and quantification of viruses in clinical settings (Garcia *et al*., 2005).

1.5.8.7 PLEX-ID™ technology

In the PLEX-ID™ system (Abbott Molecular) target sequences are initially amplified by PCR using primers targeting highly conserved regions of pathogenic microorganisms that flank variable regions. PCR products are analysed using electronspray ionization mass spectrometry (ESI-MS) to determine the base composition of the amplified products. Sequence information is matched with a known sequence database to confirm the presence of any organism. PlEX-ID™ system 95% sensitivity was observed for the detection of influenza A and B and 88% for RSV when compared with other tests such as antigen detection, culture and PCR. However, the PLEX-ID™ system was 100% sensitive for detection of adenovirus, parainfluenzavirus and coronavirus (Chen *et al*., 2008).

1.5.8.8 ProFlu-1™

Pro-Flu-1 (Pro-Flu+; Prodesse Inc.) is a FDA approved multiplex real-time RT-PCR system used for the detection of influenza A, influenza B and RSV. In two other studies the sensitivity and specificity of using ProFlu-1 assay for the

detection of influenza A was 100% and 93% and for influenza B were 98% and 99%, respectively, compared to immunofluorescence (IF) and culture assay (LeGoff *et al*., 2008; Liao *et al*., 2009).

A summary of different molecular techniques with their salient features have been presented in table 1.1 (Adapted from Olofsson *et al*., 2011).

Table 1.1: Overview of different molecular methods

Molecular method	Description	Comment
Conventional PCR	Detection: colorimetric or agarose gel electrophoresis	Quantitation: not possible Contamination: high risk Multiplexing: limited
Real-time PCR	Evaluation: colorimetric using light emitting probe	Quantitation: possible Contamination: low risk Multiplexing: limited Specific detection: possible
Multiplex PCR-based liquid array (Luminex®; Luminex Corporation); X-TAG® RVP (Abbott); ResPlex® (Qiagen); MultiCode®-PLx (EraGen Biosciences)	PCR amplicons are hybridised to coloured beads	Quantitation: not possible Multiplexing: excellent Contamination: low risk Cost: expensive
NASBA, LAMP	Amplification: isothermal Detection: colorimetric or agarose gel electrophoresis	Thermocycler: not needed Sensitivity: high Quantitation: not possible Cost: affordable
Rapid diagnostic PCR instruments: Xpert® (Cepheid) FilmArray® (Idaho) Jaguar® (BD diagnostics) Infiniti® (AutoGenomics)	System: integrated extraction and PCR system Sample run: preloaded cassettes	Hands on time: minimum Specimen batching: not required Cost: relatively expensive

1.6 Non-PCR based isothermal amplification of DNA/RNA

Isothermal amplification of nucleic acids in molecular biology has enabled scientists to amplify nucleic acids without using a thermocycler. Isothermal amplification of nucleic acid is relatively easy, cheap and can be done in remote areas where it is not possible to gain access to a thermocycler. Amplification of nucleic acids is essential in research, medicine and agriculture for the detection and characterization of genomic sequences (Moore, 2005). Although PCR-based amplification of target sequences is widespread, several non-PCR based amplification techniques have been developed in the last two decades. Development of these new isothermal amplification techniques has been possible through mimicking *in vitro* nucleic acid amplification by providing necessary proteins, dNTPs, primers and templates for synthesis of DNA/RNA.

1.6.1 Transcription mediated amplification (TMA)/ nucleic acid sequence-based amplification (NASBA)

Transcription mediated amplification (TMA) is very similar to nucleic acid sequence-based amplification (NASBA) (Compton *et al*., 1991) (Section 1.5.5). Three enzymes are used in this technique including reverse transcriptase, DNA dependent RNA polymerase and RNase H for reverse transcription and synthesis of multiple copies of RNA from the starting template RNA. NASBA can amplify 1 billion copy of the RNA complementary to the target RNA in 1.5 hour at 41º C. Amplified products can be visualized after gel electrophoresis or detected using fluorescence probes in real-time PCR amplification or colorimetric assays (Abd *et al*., 2005; Gill *et al*., 2006; Polstra *et al*., 2002). NASBA has already been approved by the Food and Drug Administration, USA for detection of HCV and HIV-1 (Guichon *et al*., 2004; Barker *et al*., 2000). NASBA amplified product was also used for real-time detection on a single chip device by Dimov *et al*. (2008).

1.6.2 Signal mediated amplification of RNA technology (SMART)

The signal mediated amplification of RNA (SMART) technology (Figure 1.2) was developed by Cytocell Ltd., Banbury, UK for diagnosis of clinical samples (Wharam *et al*., 2001). SMART technology also known as CytAMP (British BioCell International, Cardiff, UK) has been compared with conventional methods for specific detection of methicillin resistant *Staphylococcus aureus* (MRSA) (Levi *et al*., 2003). The sensitivity and specificity of CytAMP for the diagnosis and susceptibility testing of methicillin resistant *Staphylococcus aureus* (MRSA) was found to be comparable to those of PCR (Brown *et al*., 2005). It was also found that SMART produces many copies of an RNA signal at 41º C in the presence of RNA or DNA. A three-way junction (3-WJ) is formed after hybridization between the target and two probes, one contains RNA signal sequence and the other contains T7 promoter sequence (Hall *et al*., 2002). *Bst* DNA polymerase extends the short probe to generate double stranded T7 RNA polymerase promoter for subsequent synthesis of multiple copies of RNA using T7 RNA polymerase. Synthesized RNA can bind to the second probe, extended by *Bst* DNA polymerase generating double stranded promoter leading to transcription of RNA. Amplified RNA can be detected by enzyme linked oligosorbent assay (ELOSA) or real-time PCR assay (Tyagi *et al*., 1998; Tyagi and Kramer, 1996).

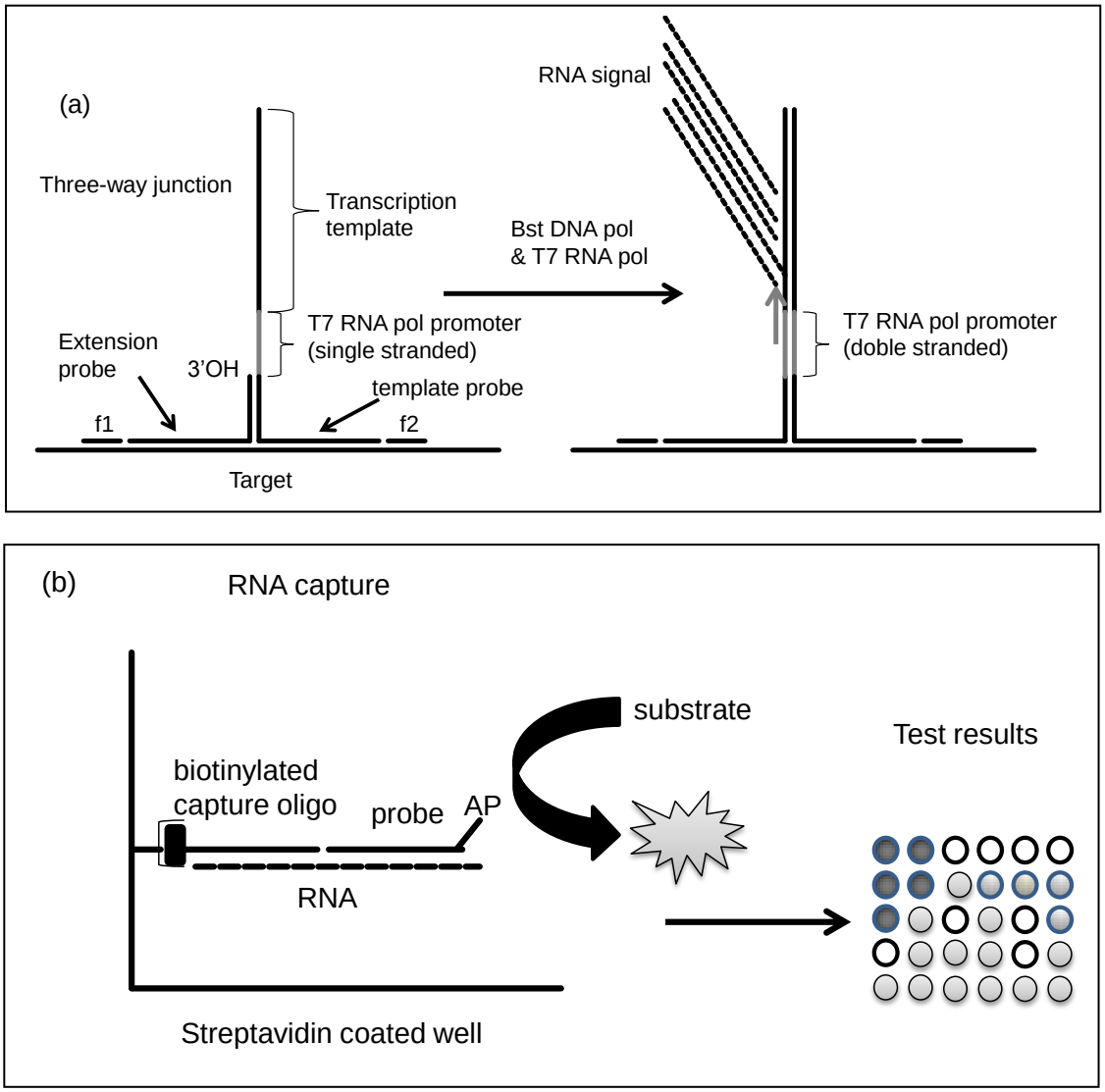

Figure 1.2: The signal mediated amplification of RNA technology (SMART). (a) Hybridization of probes to the specific target to form three way junction, synthesis of double stranded T7 RNA promoter by *Bst* DNA polymerase and synthesis of multiple copies of RNA from T7 RNA polymerase promoter (double stranded) using T7 RNA polymerase. (b) Detection of RNA signals by ELOSA (Enzyme Linked OligoSorbant Assay) using two specific probes: a biotinylated capture probe and enzyme (Alkaline phosphatase, AP) linked detection probe. Amplification of RNA determined after detection of change in the colour of the solution after adding the substrate to the reaction. Figure adapted from Wharam *et al*., 2007.

1.6.3 Strand displacement amplification (SDA)

Strand displacement amplification (SDA) uses a restriction endonuclease to nick the unmodified strand of its target DNA and an exonuclease deficient DNA polymerase to extend the 3' end at the nick and displace the downstream DNA strand. After an initial heat denaturation, four primers (B1, B2, S1 and S2) bind to the target strands flanking the sequence to be amplified. The four primers are extended simultaneously by exonuclease deficient DNA polymerase. Extension of B1 displaces the S1 primer and B2 displaces S2. SDA primers contain restriction sites and new DNA strand flanked by the nickable sites is synthesized through their extension and strand displacement activity. The newly synthesized DNA is nicked again, the 3'-end is extended, displacing a single stranded copy and regenerating double stranded DNA in the presence of excess primers. Each single stranded DNA can be amplified 10^{10}-fold using this technology. SDA amplicons can be used for real time assay (Nycz *et al*., 1998). SDA technology has been used for detection of pathogenic microorganisms such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae* using the Becton Dickinson Probe Tec ET System (BD Biosciences, Sparks, Md.) (Van Der Pol *et al*., 2001; Chan *et al*., 2000). SDA can also be used for amplification of RNA templates in RT-SDA format that will require addition of reverse transcriptase to the reaction mix (Nycz *et al*., 1998; Hellyer *et al*., 1999).

1.7 Amp-PCR: Combining phi29 amplification with RT-PCR

The sensitivity and specificity of real-time PCR for detection of any pathogen depends on several factors for example, binding of the primers to desired sequences, genome variability or mutation in the primer-binding region of the virus genome, copy number of genome and efficiency of the reaction. Sometimes, it is not possible to amplify any target sequence present in very low concentration from complex samples containing different types of templates by PCR (Karrer *et al*., 1995; Schloss *et al*., 2003). This limitation of PCR can lead to false negative results which may delay accurate diagnosis and treatment of the patient. It was

found in a study that the detection of herpes simplex virus was often missed in PCR detection when present in very low levels in cerebrospinal fluid (CSF) samples (Schloss *et al*., 2003). A new approach (Amp-PCR) has been developed recently to improve detection of samples containing as little as a single copy of a viral genome. The Amp-PCR technique combines random unbiased isothermal phi29-amplification with a virus-specific real-time PCR which enhances the PCR signal $100x10^6$-fold. Phi29 amplification enriches the template concentration preceding the PCR in a single tube reaction which makes it possible to detect amounts of template normally below the detection limit of PCR (Erlandsson *et al*., 2010).

1.8 Viral metagenomics

Metagenomics is a culture and sequence independent method to study genomic sequences in environmental and clinical samples. Metagenomic approaches can help study the genomic diversity in a specific environmental niche bypassing conventional culture techniques which have a number of limitations including isolation of the members present in nature. Metagenomic approaches to viral characterisation have been applied to seawater, near shore sediments, faeces, serum, plasma and respiratory secretions and have broadened the range of known viral diversity in these niches (Allander *et al.,* 2001; Breitbart *et al.,* 2005 and 2002; Mushahwar *et al.,* 1999). The ability to detect previously uncharacterised viruses can be improved by the selection of samples with high viral loads such as during the acute phase of infection, purification of viral particles, removal of cellular nucleic acids, efficient sequence-independent amplification of viral RNA and DNA, recognisable sequence similarities to known viral sequences and the deep sampling of the nucleic acid populations through large scale sequencing. Some animal viruses have recently been identified using sequence-independent methods (Victoria *et al.,* 2008). Viral metagenomic approaches provide novel opportunities to generate an unbiased characterisation of the viral populations in various systems and environments. The use of molecular tools is revolutionising our understanding of the composition and diversity of microbial communities. The

study of bacterial communities using 16S ribosomal RNA gene sequencing in environmental and clinical samples has led to the recognition that around 99% of bacterial species cannot be cultured and bacterial communities are much more diverse in terms of community structure than previously thought (Singh *et al*., 2009). More recently, similar observations have been made studying viral communities (Fadrosh *et al*., 2011; Cann *et al.,* 2005; Breitbart *et al.,* 2005 and 2002).

1.8.1 Molecular methods and approaches used in viral metagenomics for virus discovery

1.8.1.1 Representational difference analysis

Representational difference analysis combines subtractive hybridization with gene amplification to determine the differences between two samples. The subtractive hybridization technique is used for selective amplification of nucleic acids of pathogenic microorganisms. Nucleic acids from the infected tissue (tester) are hybridised with the uninfected (driver) nucleic acids for several rounds for separation of un-hybridised DNA. Un-hybridised single stranded tester nucleic acids are purified using hydroxyapatite chromatography (Vande Woude *et al*., 1990; Marx *et al*., 1991) and subcloned to make a library. Subcloned libraries can be screened either by immunoscreening (using appropriate serum antibodies) or by matching the sequence homology to that of specific viruses. This technique requires large amounts of starting materials and is time consuming. However, several viruses have been discovered using a subtractive hybridization technique for example, Kaposis sarcoma-associated herpesvirus (HHV8) (Chang *et al*., 1994), Torque teno virus (TTV) (Nishizawa *et al*., 1997) and GB virus A and B (Simons *et al*., 1995).

1.8.1.2 Sequence-independent nucleic acid amplification (SISPA)

Reyes *et al*., (1991) first reported the SISPA technique for the detection of unknown viral nucleic acids (Figure 1.3). Viruses containing RNA genomes are

reverse transcribed to cDNA using random primers and reverse transcriptase. Double stranded cDNA is synthesized through digestion of the RNA (using RNase H) from RNA/DNA hybrid and synthesis of the second strand using DNA polymerase activity. The resulting double stranded cDNA is digested with appropriate restriction enzymes for example, EcoR1 and Not1 and ligated to adaptor linkers complementary to the restriction sites. The DNA is digested with a suitable enzyme for example, Mbo I for the ligation of adaptor linkers complementary to the restriction enzyme cleavage sites. This adapter linker portion contains primer sequence which is subsequently used for PCR amplification of the DNA fragments flanked by the adaptor linkers. PCR amplified products are cloned in suitable vectors to make a library of the whole genome sequences. The cloned library sequences can be screened through sequencing the inserted sequences or immunoscreening of the expression clones. Immunoscreening of the expression clones subsequent to library preparation using the SISPA technique was found to be useful for the characterisation of Norwalk viruses from faeces (Matsui *et al*., 1991). The authors used the SISPA technique to amplify cDNA prepared from 10 µg of nucleic acid extracted from 1.5 grams of faecal sample for detection of Norwalk virus. In a recent study, the SISPA technique has also been used for identification and genetic characterisation of novel porcine bocaviruses (Cheng *et al*., 2010).

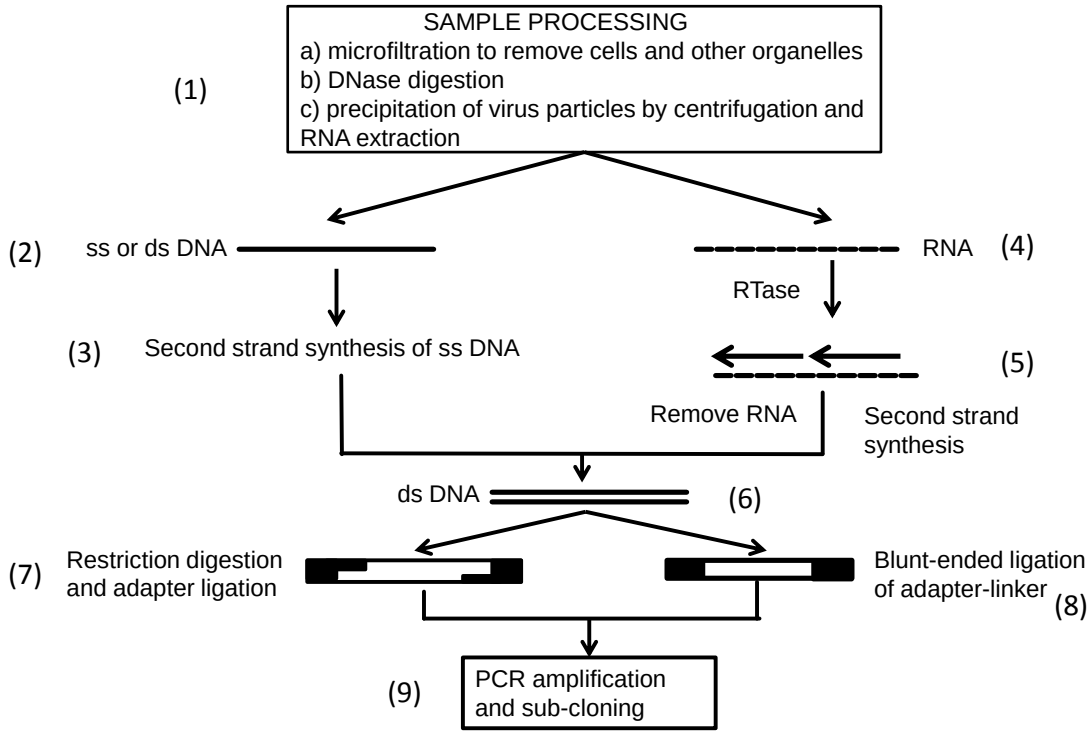

Figure 1.3: Schematic diagram of sequence independent single primer amplification (SISPA). (1) Sample is processed to remove other cells and cell free organelles and to digest cell free DNA; (2) ss or dsDNA; (3) in case of ssDNA second strnd is synthesized; (4) ssRNA; (5) RNA is reverse transcribed and the second strand is synthesized; (6) dsDNA,, (7) restriction digestion and adapter ligation; (8) ligation of adapter-linker to blunt-ended dsDNA; (9) PCR amplification from the adapter-linker and subcloning. Figure adapted from Ambrose and Clewley, 2006.

1.8.1.3 Linker amplified shotgun library (LASL)

Linker amplified shotgun library (LASL) is another method for sequence independent amplification of whole genomes. Genomic DNA is sheared at random sites using HydroShear (Genomic Solutions, Inc.). The ragged ends are repaired using T4 DNA polymerase and phosphorylated using T4 polynucleotide kinase. Linker sequences are ligated to these fragments of DNA to allow primer binding. Primers complementary to the linker sequences are used for PCR amplification of the DNA fragments flanked by the linker sequences. Viral RNA can be reverse transcribed into cDNA followed by double strand synthesis which is used for linker amplified shotgun library preparation. PCR amplified products can be sub-cloned in suitable vectors to make a genomic library (Breitbart *et al*., 2002; Cann *et al*., 2005). The LASL technique has recently been applied to the metagenomic analysis of RNA virus communities in sea water (Culley *et al*., 2006).

1.8.2 Whole genome amplification (WGA)

Whole genome amplification is very important for the amplification of nucleic acid sequences from limiting amount of samples for genomic research for example, microarray analysis and sequencing. It is challenging to amplify entire cellular DNA considering the mechanisms involved within a cell system. The cellular process for DNA replication is very complex and requires more than 20 different purified proteins to reconstitute replication of dsDNA *in vitro* (Kornberg and Baker, 1991). Unfortunately these reconstituted reactions are currently too complex to facilitate their replication in the laboratory and it has not been possible to set up complete chromosomal DNA replication as a routine laboratory technique. A number of whole genome amplification (WGA) methods have been introduced since the early 1990s to amplify genomic DNA. Broadly there are two categories of WGA available. One approach uses variations of the polymerase chain reaction (PCR) to randomly amplify the genomic sequences using random or degenerate primers (Dean *et al*., 2002; Nanda *et al*., 2008). The first PCR based WGA amplification method was called primer-extension pre-amplification (PEP) (Zhang *et al.*, 1992) and uses random primers for amplification of DNA sequence. Alternatively,

degenerate primers can be used in another method called degenerate oligonucleotide-primed PCR (DOP-PCR) (Telenius *et al.*, 1992; Cheung and Nelson, 1996). In another PCR-based whole genome amplification method genomic DNA can be shredded to small fragments, ligated to suitable adaptors and amplified using the PCR primers from the adapter sequences (Cann *et al*., 2005; Section 1.8.1.3). These PCR based amplification can generate microgram quantities of genomic-representative DNA.

The other group of methods for WGA is based on isothermal amplification of DNA by adding special types of DNA polymerases for example, phi29 or *Bst* DNA polymerase in the presence of random primers. The process of phi29 and *Bst* DNA polymerase amplification is described in Section 1.8.2.1.

1.8.2.1 Multiple displacement amplification of genomic DNA

Multiple displacement amplification (MDA) is one type of whole genome amplification method that amplifies whole genomes isothermally in a similar way to the cellular processes. The enzymes used for MDA are phi29 and *Bst* (exonuclease minus) DNA polymerases which can invade the replication fork efficiently without the help of a helicase. The former binds tightly to single stranded DNA and replicate a new complementary strand by displacing the other strand of DNA. Phi29 and *Bst* (exonuclease minus) DNA polymerase have been isolated from bacteriophage phi29 and *Bacillus stearothermophilus*, respectively. DNA is denatured either by chemical denaturation (using KOH) or by heat (95 ºC). Random hexamers, dNTPs and DNA polymerase (phi29 or *Bst*) are added to the denatured single stranded DNA for primer extension and synthesis of the complementary strand. The enzyme extends the random primers and displaces the 5’-ends of the other synthesizing strands. Thus phi29 or *Bst* (exonuclease minus) DNA polymerase can establish a new replication fork when it encounters regions of double stranded DNA. Random primers bind to the newly formed single stranded DNA and initiate synthesis of multiple copies of template DNA.

Phi29 DNA polymerase is derived from *Bacillus subtilis* phage phi29 having a processivity of about 70000 nucleotides, which means it synthesize 70000 nucleotides every time it binds to the DNA and an initiation primer. Phi29 DNA polymerase is more efficient (error rate of 1 in 10^6) than Taq DNA polymerase (error rate 1 in 10^3) and helps to reduce the incidence of artificial mutation (Esteban, 1993). *Bst* DNA polymerase (exonuclease minus) is a recombinant form of a 67 kDa DNA polymerase protein which is derived from *Bacillus stearothermophilus* DNA polymerase (large fragment). Phi29 DNA polymerase has been used for the amplification of whole genomes in previous studies more than the *Bst* DNA polymerase. Both phi29 and *Bst* DNA polymerase amplifies the whole genome in a similar mechanism. However the optimum temperature for phi29 DNA polymerase activity is 30 °C and for *Bst* DNA polymerase it is 60-65 °C.

MDA has been used to increase the supply of purified genomic DNA from very little amount of template DNA for various downstream applications for example, cloning, sequencing, hybridisation and microarray analysis. Obviously, the quality of the stored DNA to be used is critical in obtaining high quality amplified DNA. DNA can also be amplified directly from clinical and other biological samples for example, nasal swabs, blood, buffy coats, dried blood (Hosono *et al.*, 2003; Lasken *et al.*, 2004) cultured cells and tissues (Lasken *et al.*, 2004). Whole genome amplification using the MDA technique can be useful for making a metagenomic library for detection and characterisation of previously undescribed viruses. Phi29 DNA polymerase has been used successfully for amplification and detection of polyomaviruses (Johne *et al*., 2006a and 2005), anellovirus (Niel *et al*., 2005) and circoviruses (Johne *et al*., 2006b).

1.8.2.2 Multiple displacement amplification from a single bacterial cell

Multiple displacement amplification (MDA) (Dean *et al.*, 2001; Detter *et al.*, 2002) is so efficient that it can amplify genomic DNA from a single bacterial cell (Raghunathan *et al.*, 2005). A single MDA reaction starting from a single bacterium

can be amplified approximately 5 billion-fold producing 24 µg of amplified product. Moreover, such amplified DNA is suitable for downstream applications such as species identification, genotyping, and DNA sequencing. A single bacterium can be isolated by simple dilution, using flow cytometer or using fluorescent *in situ* hybridization (FISH) combined with cell isolation by microcapillary micromanipulation (Ishøy *et al.*, 2006). Cells isolated using such a technique will enable researchers an unprecedented ability to investigate the genome of a new species present in a niche with other microorganisms. MDA can be applied to such genomes (Schloss and Handelsman, 2003; Venter *et al*., 2004) to study diversity of microbial flora in clinical or environmental samples. Genomes of unculturable microorganisms can be directly amplified using MDA. This approach will complement the metagenomic shotgun approach (Venter *et al.*, 2004) of sequencing a mixture of species present in clinical or environmental samples.

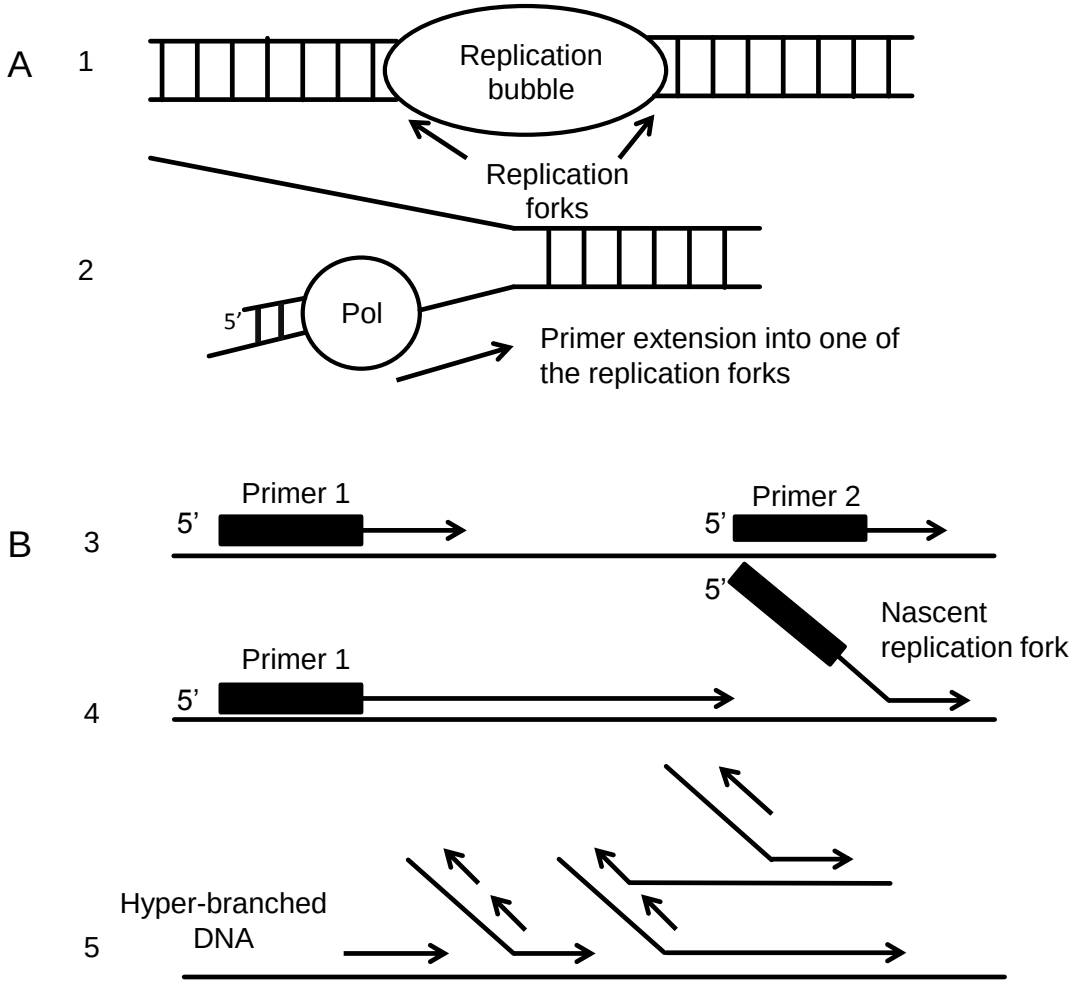

Figure 1.4 Cellular (1-2) and cell free (3-5) DNA replication processes. (A) A simplified diagram of DNA replication within the cell; (1) replication bubble is formed; (2) DNA is replicated through extension of the primers using DNA polymerase III. (B) Isothermal whole genome amplification; (3) random primers bind to the denatured template DNA; (4) primers extends (using phi29 or *Bst* DNA polymerase) and displaces the 5'-end of the other primer replicating DNA template upstream; (5) formation of hyper-branched DNA due to the random priming and extension of the random primers. Figure was adapted from Lasken *et al*., 2005.

1.8.2.3 Rolling circle amplification (RCA)

Rolling circle amplification is most useful for amplification of circular DNA viral genomes or plasmids (Breitbart, 2005; Dean *et al*., 2001). Once the phi29 DNA polymerase starts synthesizing the complementary strand in a circular DNA, it displaces its 5'-end of the sequence and continues to synthesize a new strand multiple times around the circle. It is now possible to use either sequence specific primers or random primers for RCA of circular templates. RCA using random primers is often termed as multiply-primed RCA. The primers (random or sequence specific) bind to the displaced strands and convert them to double stranded DNA (Dean *et al*., 2001). This technique can be used for generating libraries from plasmids (Dean *et al*., 2001; Detter *et al*., 2002) and circular genomes of viruses for example, human papilloma virus in a cervical keratinocyte cell line (Rector *et al*., 2004). This powerful technique has also been applied to obtain the full genome sequence of polyomaviruses (Johne *et al*., 2006 and 2005), anellovirus (Niel, 2005), circoviruses (Johne *et al*., 2006b), geminiviruses (Haible, 2006), plant begomovirus (Inoue-Nagata *et al*., 2004) and wasp polydnavirus (Espagne *et al*., 2004).

1.8.3 Purification of viral nucleic acids

Metagenomic studies of virus sequences are difficult due to the co-amplification of undesired sequences during sequence independent amplification of whole genomes using the sequence independent single primer amplification (SISPA) technique. Separation of viral particle from the other background of prokaryotic and eukaryotic nucleic acids will improve the selective amplification of viral nucleic acids. Several approaches have been used so far for example; filtration, density gradient centrifugation and enzymatic removal of non-particle protected nucleic acids for removing background sequences and selective amplification of virus sequences.

Filters having pore sizes of 160-450 nm have been used for separation of bacteria, eukaryotes and large aggregates of cells from viruses in previous studies (Allander

et al., 2005; Breitbart *et al*., 2002). Filtration works on the basis of a size exclusion mechanism allowing virus particles to pass through the membrane and sieve out larger particles. Virus particles can also be concentrated from larger volume of samples for example, sea water using tangential flow filtration technique (Breitbart *et al*., 2002; Culley *et al*., 2006 and 2003). Cesium chloride density gradient ultra-centrifugation has also been used for separation of virus particles from environmental and clinical samples (Breitbart and Rohwer, 2005).

Virus particles remain protected inside the capsid from DNase digestion. Non-particle protected cell free nucleic acids can be easily digested using enzyme such as; DNase I. Digestion of the cell free nucleic acids will improve selective amplification of viral nucleic acids. Allander *et al*., (2001) used a DNase treatment step in order to remove host DNA from serum samples prior to SISPA amplification and following this could detect new types of bovine parvoviruses.

1.8.4 Next generation sequencing (NGS)

Sanger sequencing is the original sequencing method used for determination of DNA sequences. Sanger sequencing method requires specifically labelled nucleotides and a specific primer to start read at a specific location. Different labels of each nucleotide are recorded at different levels for sequencing DNA. It is now possible to determine DNA of 1000-1200 bp through Sanger sequencing. Relatively longer DNA sequences can be determined through shotgun sequencing method. In this method genomic DNA is sheared into smaller fragments and cloned into suitable vectors. Cloned DNA fragments can be sequenced individually to determine the cloned sequences. Individual sequences can be aligned and reassembled based on the partial sequence overlap to obtain the complete DNA sequences.

Next Generation Sequencing has been developed from shotgun sequencing using the philosophy of massive parallel sequencing (Venter *et al*., 2003; Margulies *et al*., 2005; Shendure *et al*., 2005). In NGS DNA template is randomly broken to

smaller fragments and ligated to designated adapter sequences. These DNA fragments are synthesized and sequenced using a primer complementary to the adapter sequences. NGS is also termed as massively parallel sequencing. There are five types of NGS technologies commercially available for whole genome sequencing for example, Roche GS-FLX 454 Genome Sequencer, the Illumina Genome Analyzer, the ABI SOLiD analyzer, Polonator G.007 and the Helicos HeliScope platforms. The performance of these platforms for sequencing varies for the read lengths, error rates and error profiles compared to the Sanger sequencing. The read lengths for NGS are relatively shorter compared to the Sanger sequencing which can vary between 50 and 500 continuous basepair reads depending on the type of NGS platform used. The high throughput capacities of the NGS technology has increased the speed and reduced the overall cost for sequencing (Mardis, 2006, 2009; Schuster, 2008; Shendure and Ji, 2008; Ng *et al*., 2009; Tucker *et al*., 2009; Metzker, 2010).

1.9 High resolution melting (HRM) analysis

HRM analysis depends on the melting behaviour of the post-PCR amplified DNA. Most of the nucleic acid amplification and identification techniques depend on the hybridization of sequence specific primers and probes for example, in real-time PCR, fluorescent in situ hybridization (FISH) and microarray analysis (Wittwer and Kusukawa, 2005). High resolution melting (HRM) of DNA is a newer approach for detection and characterisation of PCR products by melting them in the presence of a DNA saturating dye. The DNA saturating dye (such as EvaGreen) actively binds to the double stranded DNA and emits fluorescence. The amount of fluorescence increases with the increase in DNA concentration. However, the double stranded DNA will melt to single stranded DNA at a higher temperature releasing the intercalating dye from the DNA. This will cause a gradual decrease in the fluorescence due to the release of the dye bound to the double stranded DNA. If the change in the fluorescence is plotted against the change in temperature it will produce a characteristic curve for a specific sequence (Figure 1.5). The temperature at which 50% of the DNA melts is considered as the melting

temperature (T_m) of the DNA. The value of T_m will depend on different factors for example, buffer composition and G+C content of the sequence. The T_m can be estimated using the formula; T_m = 2º C x (number of [A+T] + 4º C x (number of [G+C]). This melting pattern and the melting temperature of DNA can be utilised to determine the similarities and differences of different DNA sequences after HRM analysis. HRM analysis requires sophisticated technology for highly controlled temperature transition, data acquisition and software analysis to determine the variations in the amplified DNA. After analysis of the HRM data normalised fluorescence curves are generated for accurate detection of variations in the DNA sequences (Gundry *et al*., 2003). During normalisation, fluorescence signals generated from all samples are set within a range of 100% to 0% to help determining the similarities and differences of different sequences. The normalisation process will bring the similar sequences closer and move different sequences apart. The melting pattern of the PCR product depends on the inherent property of the DNA such as the length of DNA and GC content of the sequence. This DNA melting pattern can also vary with the other factors for example, buffer composition, DNA quality and concentration of the template DNA (Martín-Núñez *et al*., 2011; Taylor *et al*., 2001). Similar concentration of the DNA template should be used for PCR amplification to obtain similar concentrations of the amplified products so that they can be analysed using the same software with little variations in different samples. Very low concentrations of the template (C_T value >30) may produce nonspecific products which may interfere with the HRM analysis. Different nucleic acid extraction methods may vary in the concentrations of inhibitors in the extracted nucleic acid templates which may produce false positive results determining genetic variants after HRM analysis (Martín-Núñez *et al*., 2011).

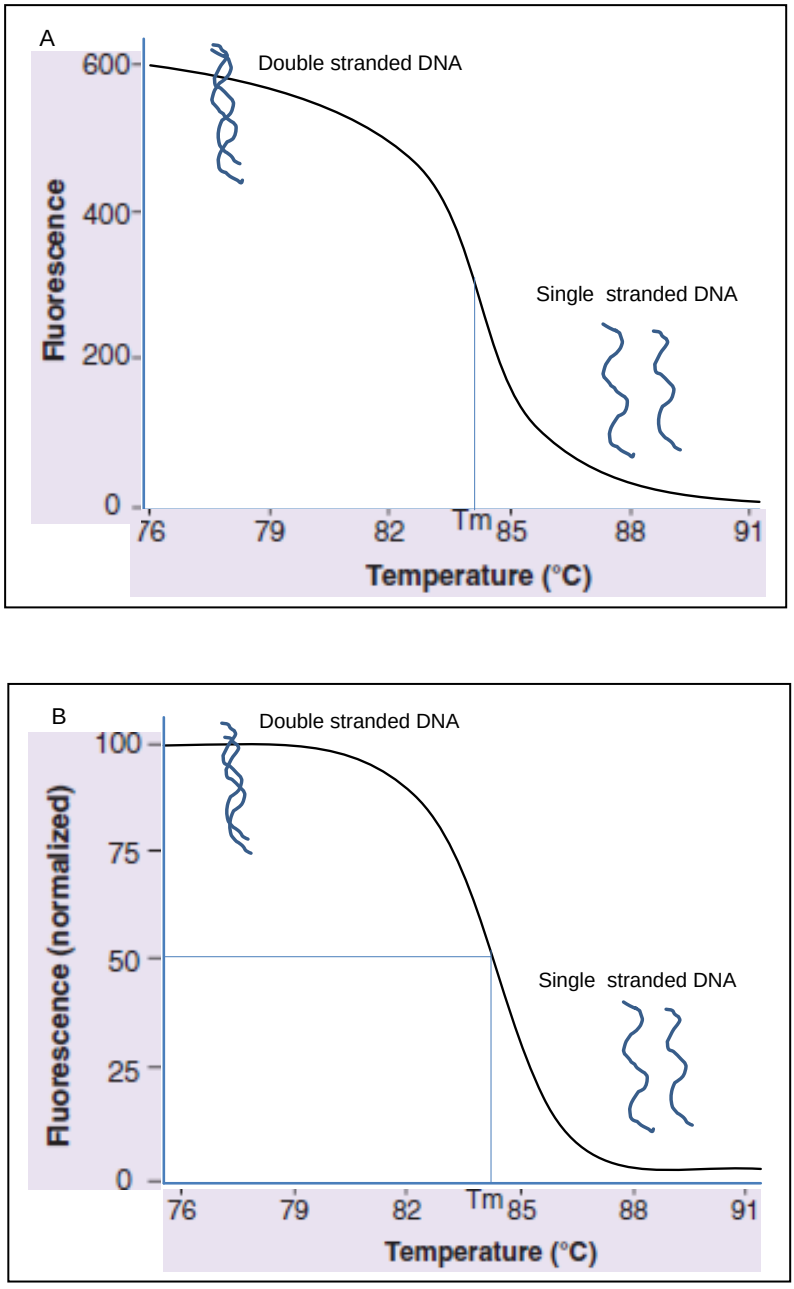

Figure 1.5: High resolution melting curve of DNA in the presence of DNA saturating dye. (A) melting curve before normalisation, (B) melting curve after normalisation. Figure adapted from Reed *et al*., 2007.

HRM technology has been applied in different types of assays including SNP genotyping, scanning for mutations in diseases and cancer related genes, DNA fingerprinting, species identification and genotyping (Martín-Núñez *et al*., 2011; Erali *et al*., 2008). HRM analysis was applied by Wittwer *et al*., (2003) for typing six common beta-globin genotypes (AA, AS, AC, SS, CC, and SC). HRM analysis was also used for the characterisation of short (38-50 bp) and large (600 bp) fragments of DNA. However, shorter fragments are more useful for accurate detection of mutations or variation in the sequences (Liew *et al*., 2004). The HRM technique has been applied for screening large fragments of DNA before sending them for sequencing.

Provaznikova *et al.*, (2008) applied HRM technology for screening myosin heavy chain 9 (MYH9) gene which avoided more than 85% of unnecessary sequencing of the MHY9 gene. The application of HRM analysis for SNP-typing is relatively cheaper and faster than PCR amplification and sequence analysis. Sensitivity of SNP typing can be increased by amplifying a smaller fragment of 80-100 bp. Addition of an unlabelled melt probe was found to be useful for determining homozygote variants in SNP typing in cancer patients (Nguyen-Dumont *et al*., 2009; Zhou *et al*., 2008). Lately, HRM analysis has been used for epigenetic study for the diagnosis of disease states for example, inherited disorders and cancer (Egger *et al*., 2004). High resolution melting analysis of the amplified DNA can easily reflect on the methylation status of the original DNA template (Dahl and Guldberg, 2007). Unlike other types of HRM analysis the epigenetic study requires a bisulphite treatment for determination of methylation pattern. Bisulphite treatment converts unmethylated cytosines to uracil in the sequence. Consequently a thymine will be incorporated instead of uracil in the DNA sequence during PCR amplification. Bisulphite treatment will not be effective on methylated cytosines which will increase the GC content in the PCR product compared to the unmethylated sequences. Consequently, post-PCR high resolution melting analysis will show the differences between methylated and unmethylated sequences. HRM analysis has been used for screening methylation in the *BRCA1*

gene promoter associated with breast cancer (Snell *et al*., 2008). HRM technology has also been used for determining the similarities and differences between different clonal sequences (Pepers *et al*., 2009).

1.10 Aims and objectives of the present study

Based on previous studies it has been observed that most respiratory tract infections are associated with viruses. A wide range of pathogens are associated with respiratory tract infections. It is difficult for even an experienced clinician to determine the causative agent of the infection based on clinical data alone therefore it is more useful to detect a panel of pathogenic microorganisms for accurate diagnosis of respiratory tract infections. Rapid developments in molecular techniques have improved the multiplex detection of pathogenic viruses of the respiratory tract. It is now possible to detect a panel of pathogenic viruses qualitatively and quantitatively in a single reaction (Sanghavi *et al*., 2011; Fan *et al*., 1998). Besides PCR-based techniques a large number of isothermal nucleic acid amplification techniques have also been introduced by different workers for multiplex detection of respiratory viruses (Wat *et al*., 2008; Lau *et al*., 2010). Loop-mediated isothermal amplification (LAMP) and reverse transcription loop-mediated isothermal amplification (RT-LAMP) have been considered as commercial tests for diagnosis of viruses (Parida, 2008). Appropriate diagnosis of respiratory viruses using molecular methods is limited by technical complexity, cost and lack of validation and standardization of assays. Interpretation of reports can also be difficult due to the frequent co-infection caused by multiple viruses in the respiratory tract (Zhang *et al*., 2011). However, the development of alternate new molecular methods for multiplex detection of respiratory viruses can help accurate detection pathogenic viruses for the control of spread and intervention of infections caused by pathogenic viruses of the respiratory tract.

In this thesis a systematic study was conducted to evaluate the isothermal amplification of nucleic acids to enrich the template concentration to enhance the molecular detection of pathogenic viruses. A novel protocol was developed by

combining reverse transcription and *Bst* DNA polymerase amplification to enrich the copy number of cDNA to increase sensitivity of molecular detection by PCR. A laboratory based study was conducted to evaluate the use of RT-Bst technique on known RNA virus genomes for example, bacteriophage MS2 and hRSV. This novel protocol was applied on seventy patient samples to evaluate the applicability of RT-Bst PCR compared to other molecular tests such as one-step RT-PCR and immunofluorescence. RT-Bst PCR was further modified for the development of a novel, low-cost and simple protocol for whole genome amplification of RNA viruses as an alternate to sequence independent single primer amplification (SISPA). Screening the whole genome amplification library using the sequencing or immunoscreening technique is laborious and expensive. In this study high resolution melting (HRM) analysis was used to demonstrate its application as an alternative and cheaper method for screening library sequences. HRM analysis was also used to demonstrate its application for rapid detection and identification of common pathogenic viruses of the respiratory tract.

Chapter 2

General Methods

2.1 Viral and bacterial stocks and patient samples used in this study

2.1.1 Viral stocks used in this study

Bacteriophage lambda (a gift from Professor Anne Moir, University of Sheffield) and MS2 (DSMZ, Braunschweig, Germany) were used as controls for DNA and RNA viruses, respectively (Mahony *et al.,* 2007). Stocks of influenza B, parainfluenza 2, human respiratory syncytial viruses (tissue culture extracts) were used as positive control samples for assays which will be described in the results section. These virus stocks were kindly provided by Jeremy Bishop, St. Mary's Hospital, Imperial College London, UK.

2.1.2 Bacterial stocks used in this study

Bacterial strains used in this study were *Staphylococcus aureus* and chemically competent *Escherichia coli,* XL1-Blue. *S. aureus* DNA was added to bacteriophage lambda DNA to mimic the undesired DNA present in clinical samples and set up assays. Commercially available chemically competent *E coli* XL1-Blue (Agilent Technology, Edinburgh, UK) cells were used for transformation of cloned plasmids (Table 2.1).

Table 2.1: Bacterial strains used in this study

Strain	Genotype/phenotype	Source
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacIqZΔ M15 Tn10 (Tet^r)]*	Agilent Technology, Edinburgh, UK
SA-1	*Staphylococcus aureus*	Laboratory stocks

2.1.3 Patient samples used in this study

Patient sample were collected from Dr Mark Atkins, Department of Virology, Chelsea and Westminster Hospital for this study after obtaining ethical approval from the University of Westminster, London, UK (App. No. 09/10/07, Date: 28/04/2010) and the NHS. For the latter, ethical approval was granted following an application via the Integrated Research Application System Proportionate Review scheme (reference 10/H0808/50). Nasopharyngeal swabs were collected by physicians during their routine diagnosis of patients reporting to hospital. Only excess residual samples were provided after being anonymised by Dr Mark Atkins as part of this study. A total of 70 samples were analysed in this study which is further described in chapter 5.

2.2 DNA, RNA and plasmid stocks used in this study

Commercially available purified DNA of bacteriophage lambda (λ) (Invitrogen, Paisley, UK or Fermentas, York, UK) and RNA of bacteriophage MS2 (Roche Applied Biosciences, Burgess Hill, UK) were used to set up different assays as described in the following chapters (Chapter 3-7). Bacteriophage λ DNA was used as a representative of double stranded DNA (48.0 kb). Reverse transcribed cDNA from MS2 RNA (3.6 kb) was used as a representative of single stranded DNA. Two plasmids pUC18 (2.6 kb) and , pKEYE (8.6 kb), provided by Dr Rebecca Smith, University of Leicester were used as representative of circular double stranded DNA.

Alternatively, carrier RNA provided with the QIAamp Viral RNA mini kit was added to a known RNA (MS2 RNA) to mimic the presence of undesired RNA in clinical samples. Specific details on how the assays were set up are elaborated in the results section.

2.3 Reagents and solutions

All solutions were prepared using deionized water produced from a Milli-Q water purification system (Millipore, Watford, UK). Solutions were sterilized by autoclaving at 121 ºC for 15 minutes. Ethanol (99.9%), isopropanol (99.5%), acetic acid (99%) and hydrochloric acid (32%) were purchased from Fisher Scientific, Loughborough, UK. The common reagents for example, ethidium bromide, sodium acetate (3.0 M, pH 5.0), ethylene diamine tetraacetic acid (0.5 M, pH8.0), 50X tris acetate and dNTPs were prepared following the protocol described in Sambrook *et al*., (1989).

2.4 Media and antibiotics

Luria-Bertani agar media containing kanamycin (30 µg ml^{-1}) was also prepared following standard methods (Sambrook *et al*., 1989).

2.5 Plaque assay and quantification of virus

Concentration of bacteriophage lambda (λ) particles in the stock was determined according to the protocol provided in the laboratory manual of Sambrook *et al*., (1989). Briefly, *Escherichia coli* XL1-Blue cells (Table 2.1) were propagated overnight at 37º C at 200 revolutions per minute (rpm) in Luria-Bertani (LB) broth. Maltose, 0.2% (w/v) and 10 mM $MgSO_4$ were added to the media to induce receptors for bacteriophage lambda particles. *E. coli* cells containing broth media were diluted to a concentration to have an optical density (OD) of 0.5 at 600 nm wavelength. An equal volume (50 µl) of *E coli* suspension and bacteriophage lambda particles were mixed, added to 10 ml of top agar media (0.8% w/v agarose) at 55º C and poured on to the Luria-Bertani agar plate. Plates were incubated overnight at 37º C to allow plaque formation. After overnight incubation at 37º C

the surface of the LB agar plate will show confluent growth of *E. coli* cells if they are not infected by any λ phage. If there is one λ phage particle it will infect and lyse *E. coli* cell and produce a clear zone of no growth on the plate, known as a plaque. The number of plaques will represent the number of bacteriophage λ particles in the tested sample.

2.6 Nucleic acid analysis

2.6.1 Agarose gel electrophoresis

Dehydrated, molecular grade agarose was obtained from Web scientific, Crewe, UK to prepare either 1.5 % (w/v) or 1% (w/v) agarose gels in 1X TAE buffer. All PCR products were run on a 1.5% (w/v) agarose gel for separation of individual band. Plasmids were electrophoresed on 1% (w/v) agarose gel to determine the size of the plasmids. Gels were stained in 1X TAE buffer containing 0.5 µg ml^{-1} ethidium bromide for 15 minutes before visualizing using a gel imaging system UVIPRO (UVITEC, Cambridge, UK). Gel images were presented in the result section either choosing normal or inverted depending on which gave the clearest image.

DNA samples were mixed with 1:5 volume of loading dye before loading into the well in the agarose gel. All PCR product sizes were determined by comparing their relative sizes with those of 100 bp DNA ladder. A 1 kb DNA ladder was used to determine the size of the plasmids in this study. Both 100 bp and 1 kb DNA ladders were obtained either from NEB, Hitchin, UK or Fermentas, York, UK.

2.6.2 Quantification of nucleic acids

2.6.2.1 Quantitation of DNA using NanoDrop™

Concentrations of different structures of DNA, purified PCR products and plasmids were quantified by measuring the optical density at 260 nm using a Nano Drop 1000 spectrophotometer (Thermo Scientific, Horsham, UK). The purity of DNA samples was determined from the ratio of the absorbance in the spectrophotometer at $A_{260nm}/A_{280nm.}$ Pure preparations of DNA have ratios between 1.8 and 2.0. In cases of any significant contamination such as from protein or phenol, the ratio will be lower.

2.6.2.2 Quantitation of amplified DNA using Picogreen dye

Quant-iT™ PicoGreen dsDNA reagents and kit (Invitrogen, Paisley, UK) were used to determine the concentration of DNA both before and after phi29 DNA polymerase amplification (Section 3.4). Picogreen dye binds only to double stranded DNA and as a result emits fluorescence (520 nm) when they are excited with light (480 nm). The amount of fluorescence emitted is proportional to the concentration of DNA in the sample. This kit contains a stock of bacteriophage λ DNA (100 µg ml^{-1}) which acts as a reference, 20X TE buffer and concentrated picogreen dye for preparation of standard curves.

Bacteriophage λ DNA was diluted at concentration of 400 ng ml^{-1}, 200 ng ml^{-1}, 100 ng ml^{-1}, 50 ng ml^{-1} and 25 ng ml^{-1} in 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) provided with the kit. Picogreen dye was freshly prepared by diluting (1:200) in 1X TE buffer. An equal volume (50 µl) of λ DNA was added to picogreen dye in a MicroWell plate (Fisher Scientific, Loughborough, UK). The fluorescence generated from the sample in the MicroWell plate (Fisher Scientific, Loughborough, UK) was measured in a fluorescence microplate reader. The samples were excited

at 480 nm to determine the fluorescence emission at 520 nm using spectrophotometer. The fluorescence reading of the dilution series of λ DNA was plotted against their respective concentrations to obtain a standard curve. The fluorescence reading of test samples was plotted on the standard curve to determine their concentration.

2.6.3 Extraction of virus genomes

Bacteriophage lambda DNA was extracted using the QIAamp DNA Mini kit (QIAGEN, Crawley, UK). Briefly, 100 µl of sample was mixed with 100 µl of lysis buffer (AL), incubated for 10 minutes at room temperature and 100 µl of ethanol (96-100%) was added and mixed gently for 15 seconds. This mixture was loaded into a spin column and centrifuged at 6000 x *g* for 1 minute to bind DNA to the column. This column was washed by passing 500 µl of wash buffer AW1 and AW2 at 6000 x *g* for 1 minute and 20000 x *g* for 3 minute respectively. DNA was eluted with 100 µl of AE buffer.

Viral RNA was extracted with either the QIAamp DNA Mini kit or QIAamp Viral RNA Mini Kit (QIAGEN, Crawley, UK). Carrier RNA (100 nanograms) was added to 400 µl of buffer AVL either from the provided RNA in the QIAamp Viral RNA Mini Kit or commercially available MS2 RNA (Roche Applied Biosciences, Burgess Hill, UK). Carrier RNA was added to the buffer in order to protect RNA from degradation and improve the binding and recovery of small amount of RNA from the column. 100 µl of nasopharyngeal sample was mixed with buffer AVL containing carrier RNA, incubated for 10 minutes at room temperature and mixed with ethanol (96-100%). This mixture was loaded into a QIAamp Mini column and centrifuged at 6000 x *g* for 1 minute to bind RNA to the column. This column was washed by passing 500 µl of buffer AW1 centrifuging at 6000 x *g* for 1 minute. This column was washed again with buffer AW2 centrifuging at 20000 x *g* for 3 minutes. RNA was eluted with 60 µl of AVE buffer.

Extracted nucleic acids were used immediately either for PCR, one step RT-PCR or RT-*Bst* amplification (Section 2.6.6). Any remaining nucleic acids were aliquoted into PCR tubes and stored at -80 ºC for future use.

2.6.4 Reverse transcription and preparation of cDNA

Complementary DNA was prepared from commercially available MS2 RNA (Roche Applied Biosciences, Burgess Hill, UK), using the 1st strand synthesis kit (NEB, Hitchin, UK) or QuanTitect™ reverse transcription kit (QIAGEN, Crawley, UK).

For 1st strand synthesis kit (NEB, Hitchin, UK), 5 µl of RNA was mixed with 1 µl of random primer (oligo dN_9, 15 µM) and 2 µl of dNTP mix (2.5 mM each), heated for 5 minutes at 70° C and cooled on ice for denaturation of RNA and hybridisation of the primers. 1 µl of 10X RT buffer, 0.5 µl of RNase inhibitor (10 units μl^{-1}) and 0.5 µl of M-MuLV reverse transcriptase (25 units μl^{-1}) were added to 8 µl of denatured RNA from previous step and incubated at 42° C for 1 hour. Enzymes were inactivated at 95° C for 5 minutes.

In case of QuantiTect reverse transcription kit 7 µl of RNA was added to 0.5 µl of Quantiscript reverse transcriptase, 2 µl of 5X Quantiscript RT buffer and 0.5 µl of RT primer mix and incubated for 15 minutes at 42° C. The reaction was inactivated at 95° C for 3 minutes.

Freshly prepared cDNA was used for PCR amplification and any excess was aliquoted into PCR tubes and stored at -80° C for future use.

2.6.5 OneStep™ RT-PCR

Reverse transcription for preparation of cDNA from RNA and PCR amplification of target sequences are two separate reactions. If these two steps are performed

separately, it is called two-step RT-PCR. On the other hand if reverse transcription and PCR amplification are combined in the same tube it is called one-step RT-PCR. The OneStep™ RT-PCR kit (QIAGEN, Crawley, UK) was used for detection of RNA viruses in this study.

2.6.5.1 OneStep™ RT-PCR (multiplex)

Three multiplex one step RT-PCR reactions were set up for detection of 12 common pathogenic viruses of the respiratory tract (Table 2.4). Multiplex 1 was set up for hRSV, influenza A, influenza B and hMPV; multiplex 2 was set up for parainfluenza 1-4; multiplex 3 was set up for hRV, HCoV 229E, HCoV OC43 and influenza C respectively. In case of multiplex reactions equimolar concentration of four sets of primers were added for detection of 4 viruses in multiplex 1, 2 and 3. An additional set of RT-PCR reactions was set up for detection of an internal control, MS2 RNA. Primer sets were selected from published papers (Table 2.4) and checked by bioinformatics analysis before use. The reaction mix was set up (Bellau-Pujol *et al.,* 2005) as described in the following table 2.2;

Table 2.2: One-step RT-PCR mix for multiplex 1, 2 and 3

Reaction component	Multiplex 1 and 3*	Multiplex 2*
Nuclease free water	0.5 µl	2.3 µl
5X RT buffer	3.0 µl	3.0 µl
dNTP (10 mM each)	0.6 µl	0.6 µl
Primer, forward 10 µM (0.75x4=)	3.0 µl	3.0 µl
Primer, reverse 10 µM (0.75x4=)	3.0 µl	3.0 µl
One step RT enzyme mix	0.6 µl	0.6 µl
Q solution	1.8 µl	0.0 µl
RNA	2.5 µl	2.5 µl
Total=	15.0 µl	15.0 µl

*Primer sets were selected from table 2.4 for multiplex 1, 2 and 3

PCR cycling: RT-PCR was initiated with 30 minutes at 50 ºC with reverse transcription reaction step followed by heat activation of hot start Taq polymerase at 94 ºC for 15 minutes. Then the thermal cycler was programed for 40 cycles, 94 ºC for 30 seconds, 55 ºC for 30 seconds and 72 ºC for 1 minute. A final extension step was performed at 72 ºC for 10 minutes. The annealing temperature for multiplex 1, 2 and internal control was 55 ºC for 30 seconds whereas annealing temperature for multiplex 3 was 58 ºC for 30 seconds (Bellau-Pujol *et al.*, 2005).

2.6.5.2 One-step RT-PCR (singleplex):

One step RT-PCR reactions (singleplex) were set up according to table 2.3 for amplification and detection of internal control MS2 RNA or any single virus. If any reaction was positive for virus sequences after multiplex one-step RT-PCR amplification, the assay was repeated using one set of primers corresponding to each virus in a singleplex one-step RT-PCR reaction.

Table 2.3: One step RT-PCR mix (singleplex)

Reaction component	Reaction volume
Nuclease free water	6.8 µl
5X RT buffer	3.0 µl
dNTP (10 mM each)	0.6 µl
Primer, forward 10 µM	0.75 µl
Primer, reverse 10 µM	0.75 µl
One step RT enzyme mix	0.6 µl
Q solution	1.8 µl
RNA	2.5 µl
Total=	15.0 µl

Table 2.4: Primers used for multiplex 1, 2 and 3

Virus	Primer name	Sequence (5'→3')	Gene	Amplicon size (bp)	Reference
Multiplex-1					
hRSV	vrs P1	5'-GGAACAAGTTGTTGAGGTT TATGAATATGC-3'	Nucleocapsid	279	Cane and Pringle (1991)
	vrs P2	5'-TTCTGCTGTCAAGTCTAGT ACACTGTAGT-3'			
Influenza A	mia 1	5'-CAGAGACTTGAAGATGTCT TTGCTGG-3'	Matrix protein	212	Donofrio *et al.* (1992)
	mia 2	5'-GCTCTGTCCATGTTATTTG-3'			
Influenza B	Mib 1	5'-AAAATTACATGTTGGTTCG GTG-3'	Matrix protein	362	Donofrio *et al.* (1992)
	Mib 2	5'-AGCGTTCCTAGTTTTACT TG-3'			
hMPV	hmpv1	5'-CCCTTTGTTTCAGGCCAA-3'	Matrix protein	416	Donofrio *et al*. (1992)
	hmpv2	5'-GCAGCTTCAACAGTAGCTG-3'			
Multiplex 2					
Parainfluenza 1	PIS1+	5'-CCGGTAATTTCTCATACCT ATG-3'	Haemagglutinin-Neuraminidase	317	Echevarria *et al*. (1998)
	PIS1-	5'-CCTTGGAGCGGAGTTGTT AAG-3'			
Parainfluenza 2	PIP2+	5'-AACAATCTGCTGCAGCAT TT-3'	Haemagglutinin-Neuraminidase	507	Echevarria *et al.* (1998)
	PIP2-	5'-ATGTCAGACAATGGGCAA AT-3'			
Parainfluenza 3	Para 3.1	5'-CTCGAGGTTGTCAGGATA TAG-3'	Haemagglutinin-Neuraminidase	189	Karron *et al.* (1994)
	Para 3.2	5'-CTTTGGGAGTTGAACACAG TT-3'			
Parainfluenza 4	PIP4+	5'-CTGAACGGTTGCATTCAG GT-3'	Phosphoprotein	451	Aguilar *et al.* (2000)
	PIP4-	5'-TTGCATCAAGAATGAGTC CT-3'			
Multiplex 3					
h RV	SRHI1	5'-GCATCIGGYARYTTCCACC ACCANCC-3'	VP4/VP2/5'NC	549	Savolainen *et al*. (2002)
	SRHI2	5'-GGGACCAACTACTTTGGG TGTCCGTGT-3'			
HCoV 229E	MD1	5'-TGGCCCCATTAAAAATGT GT-3'	Gene M	573	Vabret *et al.* (2001)
	MD3	5'-CCTGAACACCTGAAGCCA AT-3'			
HCoV OC43	MF1	5'-GGCTTATGTGGCCCCTTA CT-3'	Gene M	335	Vabret *et al*. (2001)
	MF3	5'-GGCAAATCTGCCCAAGAA TA-3'			
Influenza C	CHAA	5'-CHAAACACTTCCAACCCAA TTTGG-3'	Haemagglutinin-esterase	485	Zhang and Evans (1991)
	CHAD	5'-CCTGACAGCAACTCCCTC AT-3'			
Internal controls					
GAPDH	GAPDH1	5'-TCATCCATGACA ACTTTGGTATCGTG-3'	GAPDH	564	Gueudin *et al*. (2003)
	GAPDH2	5'-CTCTTCCTCTTGTGCTCT TG-3'			
MS2	MS2-F	5'-CTGGGCAATAGTCAAA-3'	MS2 genome	314	Dreier *et al*. (2005)
	MS2-R	5'-CGTGGATCTGACATAC-3'			

2.6.6 Nucleic acid amplification

2.6.6.1 RT-Bst amplification

RT-Bst is a novel protocol developed in this study by combining reverse transcription and *Bst* DNA polymerase amplification in the same reaction. Unlike other reverse transcription reactions, RT-Bst will not only synthesize cDNA from RNA template but also amplify it. It is envisaged that RT-Bst amplification of cDNA will amplify the template (cDNA) concentrations above the normal detection level of PCR methods used in the diagnostic laboratories. This will possibly improve the sensitivity of PCR detection of samples containing very low concentration of RNA template (under the normal detection limit). RT-Bst amplification was carried out according to Table 2.5. RNase inhibitor and premium reverse transcriptase was obtained from Fermentas, York, UK. *Bst* DNA polymerase was obtained from Cambridge Biosciences, Cambridge, UK.

Table 2.5: RT-Bst reaction mix for preparation of cDNA

Reaction component	Reaction volume
Nuclease free water	3.85 µl
10X RT buffer	1.0 µl
N15 random primer (10 µM)	1.0 µl
dNTP (10 mM each)	0.5 µl
RNase inhibitor (40.0 U μl^{-1})	0.25 µl
Premium reverse transcriptase (200 U μl^{-1})	0.5 µl
Bst DNA polymerase (8.0 U μl^{-1})	0.4 µl
Template RNA	2.5 µl
Total=	10.0 µl

The RT-Bst reaction was incubated at 25 ºC for 10 minutes for hybridization of the random primer, 50 ºC for 30 minutes for reverse transcription and 60 ºC for 1 hour

for *Bst* DNA polymerase amplification. Following this, the reaction was heat inactivated at 85 ºC for 5 minutes. After RT-Bst amplification 1 to 2 µl of cDNA was added to 20 µl of hot start Taq PCR reaction as described in section 2.6.7 for detection of viruses for multiplex 1, 2, 3 and internal control (MS2 RNA).

2.6.6.2 REPLIg amplification of DNA

Nucleic acids were amplified using the REPLIg kit (QIAGEN, Crawley, UK) following the protocol provided by the manufacturer. Briefly 2.5 µl of DNA sample was added to 2.5 µl of D1 (denaturation) buffer, mixed gently, centrifuged briefly and incubated at room temperature for 3 minutes. 5 µl of N1 (neutralization) buffer was added to the sample, mixed gently and centrifuged briefly. 40 µl of DNA polymerase master mix was added to the 10 µl of denatured DNA and incubated at 30 ºC for 16 hours using a thermocycler. REPLIg DNA polymerase was inactivated at 65º C for 3 minutes. REPLIg amplicons were diluted 1:20 and 2 µl was added as template in 20 µl of PCR reaction.

2.6.7 HotStarTaq™ PCR

HotStarTaq™ PCR reaction was used for multiplex detection of 12 respiratory tract viruses from RT-Bst amplified products in 3 multiplex reactions to compare this result with multiplex one-step RT-PCR reaction for any improvement in detection. Multiplex 1 was set up for hRSV, influenza A, influenza B and hMPV; multiplex 2 was set up for parainfluenza 1-4; multiplex 3 was set up for hRV, HCoV 229E, HCoV OC43 and influenza C. In case of multiplex reactions equimolar concentrations of four sets of primers were added for detection of 4 viruses in each multiplex 1, 2 and 3. An additional set of HotStarTaq™ PCR reactions was set up for detection of an internal control, MS2 RNA. Primer sets were selected from

published papers (Table 2.4) and the reaction mix (Bellau-Pujol *et al.,* 2005) was set up as described in Table 2.6. HotStarTaq™ polymerase was obtained from QIAGEN, Crawley, UK.

2.6.7.1 Multiplex HotStarTaq™ PCR reactions

Multiplex HotStarTaq™ PCR was set up for detection of 12 common respiratory tract viruses in three separate multiplex reactions using RT-Bst amplified product as template (Section 2.6.7). Primer sets were selected from published papers (Table 2.4) and the reaction mix was set up as described in tables 2.6 and 2.7.

Table 2.6: HotStarTaq™ PCR mix for multiplex 1, 2 and 3

Reaction component	Multiplex 1 and 3*	Multiplex 2*
Nuclease free water	3.9 µl	6.3 µl
10X PCR buffer	2.0 µl	2.0 µl
$MgCl_2$ (15 mM)	1.2 µl	1.2 µl
dNTP (10 mM each)	0.4 µl	0.4 µl
Primer, forward 10 µM (1.0x4=)	4.0 µl	4.0 µl
Primer, reverse 10 µM (1.0x4=)	4.0 µl	4.0 µl
HotStarTaq™ DNA polymerase (5.0 U μl^{-1})	0.1 µl	0.1 µl
Q solution	2.4 µl	0.0 µl
Template	2.0 µl	2.0 µl
Total=	20.0 µl	20.0 µl

*Primer sets were selected from table 2.5.1.3 for multiplex 1, 2 and 3

PCR cycling: PCR was initiated by heat activation of HotStarTaq™ polymerase at 94 ºC for 15 minutes. Then the thermal cycler was programed for 40 cycles, 94 ºC for 30 seconds, 55 ºC for 30 seconds or 58 ºC for 30 seconds and 72 ºC for 1 minute. A final extension was performed at 72 ºC for 10 minutes. The annealing

temperature for multiplex 1, 2 and internal control was 55 ºC for 30 seconds whereas annealing temperature for multiplex 3 was 58 ºC for 30 seconds (Bellau-Pujol *et al.*, 2005).

2.6.7.2 HotStarTaq™ PCR (singleplex)

Singleplex HotStarTaq™ - PCR reaction was set up for detection of the internal control, MS2 RNA using RT-Bst amplified product as template (Table 2.7). In cases when more than one virus appeared positive in multiplex HotStarTaq™ PCR, detection of each virus was repeated using singleplex HotStarTaq™ PCR using single primer sets for each virus. This singleplex HotStarTaq™ PCR reaction was also used for all other PCR reactions for setting up assays in chapter 3-6, unless stated otherwise.

Table 2.7: HotStarTaq™ PCR mix (singleplex)

Reaction component	**Reaction volume**
Nuclease free water	9.9 µl
10X PCR buffer	2.0 µl
$MgCl_2$ (15 mM)	1.2 µl
dNTP (10 mM each)	0.4 µl
Primer, forward 10 µM	1.0 µl
Primer, reverse 10 µM	1.0 µl
HotStarTaq™ DNA polymerase	0.1 µl
Q solution	2.4 µl
Template	2.0 µl
Total=	20.0 µl

PCR cycling: PCR was started with heat activation of HotStarTaq™ polymerase at 94 ºC for 15 minutes. Then the cycler was set for 40 cycles, 94 ºC for 30 seconds, 55 ºC for 30 seconds and 72 ºC for 1 minute. Final extension was 72 ºC for 10 minutes.

2.6.8 Cloning PCR products using the TOPO TA® cloning kit

PCR products were prepared for TA cloning by including a final extension step at 72 ºC for 7-10 minutes after the PCR cycle to generate a 3' adenine overhangs. On the other hand linearized vectors supplied with the kit contain 3' deoxythymidine (T) residues. This will allow PCR inserts to ligate efficiently with the vector with Topoisomerase I. PCR products were purified (Section 2.6.11) before TA cloning. TOPO TA® cloning was performed using the TOPO TA cloning kit (Invitrogen, Paisley, UK). Briefly, 4 µl of purified PCR product was mixed with 1 µl of salt solution and 1 µl of TOPO® vector and incubated at room temperature for 5 minutes for cloning the PCR insert. Chemically competent *Escherichia coli* XL1-Blue (Agilent Technology, Edinburgh, UK) cells were used for transformation of this cloned product. A total of 2 µl of TOPO cloning reaction was added to 40 µl of chemically competent *Escherichia coli* XL1-Blue cells and incubated on ice for 30 minutes. The tube containing cells was heated briefly for 45 seconds in a heating block at 42º C and immediately transferred to ice. A total of 200 µl of LB broth was added to the cell mixture and incubated at 37º C for 1 hour at 200 revolutions per minute (rpm). A total of 50 µl of broth was spread on LB agar plates containing kanamycin (30 µg ml^{-1}). Plates were incubated at 37º C overnight to allow the transformed cells to grow. Colonies were picked randomly and screened for the presence of the insert by colony PCR (Section 2.6.9).

2.6.9 Colony PCR

Colony PCR technique was used to screen colonies from kanamycin LB agar plates for confirmation of cloning and transformation. A portion of a bacterial colony was resuspended in 200 µl of sterile distilled water and heated at 95 ºC for 15 minutes. Heated cells were then centrifuged for 5 minutes at 20000 *g* to pellet the debris. Finally, 2 µl of supernatant was used as template for PCR reaction (Section 2.6.7.2). Colonies showing the predicted size of the PCR products were further analysed by plasmid extraction (Section 2.6.10) and sequencing (Section 2.7).

2.6.10 Plasmid extraction

Colonies which gave the anticipated sized product by PCR (Section 2.6.9) were propagated in LB broth containing kanamycin (30 µg μl^{-1}) overnight at 37 ºC at 250 revolutions per minute. LB broth containing the cells was used for plasmid extraction using the PureLink™ Quick Plasmid Miniprep Kit (Invitrogen, Paisley, UK). Briefly, 1-5 ml of overnight culture was centrifuged at 20000 *g* to pellet cells and remove all medium from cell pellet. Cell pellet was suspended completely in resuspension buffer (B3) with RNase A. 250 µl of lysis buffer (L7) was added to cells and mixed gently and incubated for 5 minutes at room temperature. A total of 350 µl of precipitation buffer was added and mixed thoroughly to produce a homogenous solution. The mixture was centrifuged at 12000 *g* for 10 minutes at room temperature to clarify the lysate from debris. Supernatant was loaded into a spin column and centrifuged at 12000 *g* for 1 minute to bind the plasmid to the column. The spin column was washed with 700 µl wash buffer (W9) by loading and centrifuging the column at 12000 *g* for 1 minute. The column was centrifuged for additional 1 minute at 12000 *g* to remove any residual wash buffer. Plasmid was eluted with 50 µl elution buffer (Tris-HCl, pH 8.5) by centrifuging at 12000 *g* for 1 minute. Purified plasmids were sequenced as described in section 2.7.

2.6.11 PCR product purification

PCR products were purified using QIA-quick PCR purification kit (QIAGEN, Crawley, UK) following the manufacturer's instructions. Briefly, 100 µl of buffer PB were added to 20 µl of PCR product and mixed gently. A total of 10 µl of 3 M sodium acetate was added to the mixture in order to drop the pH of the solution <7.5. An acidic pH of the solution will improve binding of the DNA to the column. The change in pH can be determined from the change in the colour of the solution to yellow. The solution was passed through the QIAquick column by centrifuging for 30-60s so that the DNA can bind to the column. Columns were washed with 750 µl of PE buffer to remove any contaminants or inhibitors from the solution. DNA was eluted from the column with 50 µl of EB buffer. Purified PCR products were either used for cloning (Section 2.6.8) or sent for DNA sequencing (Section 2.7).

2.6.12 Gel extraction of PCR product

Individual PCR bands were excised from the gel using a scalpel and purified using the QIAquick gel extraction kit (QIAGEN, Crawley, UK) following the protocol provided by the manufacturer. Briefly, DNA band was excised from the agarose gel using a clean scalpel and weighed in an electronic balance. 3 volume of QX1 buffer and 2 volume of H_2O were added to the gel containing DNA. 10 µl of QIAEX II particle was added to the solution. The agarose was dissolved in the buffer QX1 by heating at 50 °C for 10 minutes and vortexing the tube. 10 µl of 3M sodium acetate was added to the solution to reduce the pH to below 7.5. An acidic pH helps binding the DNA to the QIAEX II particles. The sample was centrifuged for 30 seconds to pellet the QIAEX II particle from the solution. The pellet was washed with 500 µl of QX1 and 500 µl of PE buffers and dried for 10-15 minutes at room temperature followed by elution in 20 µl of 10 mM Tris-HCl, pH 8.5.

2.7 DNA sequencing

Prepaid barcodes were used to send DNA samples to GATC Biotech Ltd. (London, UK) for Sanger sequencing at. 20 µl of purified PCR product (20 ng μl^{-1}) or purified plasmid containing the insert (100 ng μl^{-1}) was sent in 1.5 ml eppendorf tube to the collection point of GATC Biotech, London. Either forward or reverse primer (20 µl) corresponding to the PCR product was sent with the purified PCR product for sequencing. M13 forward (5'-TGTAAAACGACGGCCAGT-3') or M13 reverse (5'-CAGGAAACAGCTATGACC-3') primers were selected from the GATC website for sequencing purified plasmids.

2.8 Sequence analysis

Any DNA sequence received from GATC Biotech Ltd was further analysed using the Basic Local Alignment Search Tool for nucleotide (BLASTn) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) available in the NCBI sequence database to determine sequence homology. The BLASTn tool searches the nucleotide database using an internet-based nucleotide query. Once the query is sent, the program searches the NCBI database and servers, and the results are posted in the same browser window in the chosen format. The window will show some results for example, maximum score, total score, query coverage, maximum identity and the expect (E) value. It is possible to determine by how much the query sequence is similar to other sequences in the existing database after such analysis. E value is a statistical value generated after the search for similarity of sequences. The E value is also considered as the statistical significance of a match. A default threshold for matching the sequence against the database can be set during the sequence search which is 10. The default value for expect threshold is 10 because it is highly unlikely to find such 10 matches by chance. In BLASTn analysis the lower the E value the more significant the sequence similarity is.

Similar sequences were further analysed using multiple sequence alignment program CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to determine the identity amongst the similar sequences. In this analysis DNA sequences are entered in one of the supported format for example, FASTA format having annotated properly. Nucleotide sequences are submitted online for multiple sequence alignment of the similar sequences. Sequence analysis results of 10 clones selected from the whole genome library prepared from MS2 RNA and tissue culture sample positive for hRSV and influenza B using CLUSTALW are shown in chapter 6.

Chapter 3

Evaluation of phi29-amplification of different structural conformations of DNA

3.1 Introduction and aims

The recent development in molecular biology has revolutionized the clinical diagnosis of infectious diseases. Nucleic acid-based detection of pathogenic microorganisms has been shown to be more sensitive and specific compared to traditional methods (Templeton *et al.,* 2004). Many new virus types have also been discovered or characterized in the last two decades with the advent of molecular techniques. For example, Allander *et al*. (2007) identified human polyomavirus using a sequence independent single primer amplification (SISPA) technique and Lambden *et al*., (1992) detected a non-cultivatable human rotavirus using the SISPA technique. Extraction of nucleic acids from clinical samples is a bottleneck for the detection of specific sequences by PCR. The nucleic acid extraction process is usually rigorous, lengthy and involves multiple steps such as, cell lysis using alkali and/or heating, centrifugation to separate cell debris and column purification. The nucleic acid extraction process is also associated with significant loss of template and integrity of the nucleic acids of pathogenic microorganisms present in the original sample. This reduced concentration and poor integrity of nucleic acids often leads to lower sensitivity of detection of any pathogenic microorganisms by PCR (Wilson, 1997).

PCR is a very sensitive molecular method which can be used for the specific detection of pathogenic microorganisms (Vosberg, 1989). However, very low copy numbers of nucleic acids at femtogram levels are often not detected by PCR from complex samples such as samples containing various types of cDNA due to the "Monte Carlo effect" (Karrer *et al*., 1995; Schloss *et al*., 2003). This is defined as an inherent limitation of the PCR technique for detection of a target sequence from small amount of any complex template such as samples containing different types and sizes of cDNA. This limitation of PCR arises due to small and random differences in amplification efficiency between individual templates in the amplifying reaction, leading to false negative results. Amplification of nucleic acid from very low concentrations of template using whole genome amplification

techniques (Section 1.8.2) may alleviate these problems by increasing the template concentration for subsequent detection by PCR.

There are broadly two types of whole genome amplification methods available for amplification of nucleic acids, PCR and non-PCR based techniques (Section 1.5.8 and 1.6). PCR-based amplification is often biased; preferentially amplifying some portions of the genomes over others, compared to non-PCR based whole genome amplification (Hosono *et al*., 2003; Dean *et al*., 2002). Certain factors may contribute towards amplification bias, such as priming efficiency, template accessibility and GC content (Dean *et al*., 2002). Moreover, most of the PCR-based whole genome amplified products contains amplification artefacts due to the non-specific priming such as from degenerate primers and amplification of non-specific DNA (Cheung and Nelson, 1996). In a previous study, Dean *et al*. (2002) amplified a human genome using degenerate oligonucleotide-primed (DOP) PCR and multiple displacement amplification (MDA) method (Section 1.8.2) to compare the amplification bias, loss of certain loci during amplification, using these PCR-based and non-PCR based whole genome amplification techniques. Amplification of different loci of the human genome was determined by a quantitative real-time PCR technique and compared with that of unamplified human genome. Amplification bias or drop out of certain loci after this amplification was less than 3-fold for non-PCR based amplification whereas 10^4-10^6-fold for a PCR-based whole genome amplification method (Dean *et al*., 2002). In isothermal amplification phi29 DNA polymerase amplifies the whole genome uniformly. This enzyme has unique strand displacement activity and efficiency producing only 1 error per 10^6-10^7 bases (Esteban *et al*., 1993).

Phi29-amplification of nucleic acids has also been found to be useful for subsequent microarray analysis for the detection of both DNA and RNA viruses in clinical samples received for routine diagnostic analysis (Erlandsson *et al*., 2011). Jaing *et al*. (2008) reported a high density NimbleGen™ microarray system for the detection of specific genes related to microbial antibiotic resistance and virulence

mechanisms. The authors could detect these genetic characteristics of bacteria from femtogram concentrations of DNA after whole genome amplification using the REPLIg kit (QIAGEN, Crawley, UK) and microarray analysis. Wu *et al*. (2006) amplified the whole community microbial genome from picogram quantities using phi29 DNA polymerase for subsequent detection of microbial diversity by microarray analysis from groundwater contaminated with uranium and other metals. The authors found that the diversity of microorganisms containing genes involved in degradation of contaminants was greatly reduced in highly contaminated environments. It is now theoretically possible to detect pathogenic viruses, as well as previously undescribed viruses types within existing virus families using microarray platforms (Berthet *et al*., 2008; Palacios *et al*., 2007; Wang *et al*., 2002). Some of these microarray platforms have been used for the detection of viruses in respiratory samples (Berthet *et al*., 2008; Palacios *et al*., 2007; Wang *et al*., 2002; Kistler *et al*., 2007). In a recent study, Gardner *et al*. (2010) designed a pan-microbial detection array for detection of viruses (including bacteriophages), bacteria and plasmids using a novel statistical method. The authors designed an array containing larger numbers of family specific nucleic acid probes per sequence, 50 or more for viruses, 15 or more for bacteria compared to 2-10 probes per target in previous array based on the recent sequence database. They were able to detect and characterise multiple viruses, bacterophage and bacteria in faecal, serum and respiratory samples to the family and species level using this new technology.

One of the objectives of this thesis was to improve the detection of pathogenic viruses of the respiratory tract through nucleic acid amplification using phi29 DNA polymerase. The rationale for nucleic acid amplification (Section 1.8.2) was that such amplification produces large quantities of DNA from a very minute amount of sample (Hosono *et al.,* 2003). Multiple displacement amplification (Section 1.8.2) is a cell-free whole genome amplification system which uses random primers and phi29 DNA polymerase and amplifies DNA from femtogram to microgram levels using strand displacement activity (Jaing *et al*. 2008) (Section 1.8.2). This cell-free

system of phi-29 amplification will eliminate the necessity of propagation of cells and viruses in larger volumes for the amplification of nucleic acids. Phi29-amplification using a two-step priming reaction was successfully used to amplify and detect 25 picogram of circular DNA from *Borrelia burgdorferi* B31 amongst vast excess of human DNA (Xu *et al.*, 2008). Phi29 DNA polymerase has also been used for multiple displacement amplification (MDA) (Section 1.8.2) of template DNA from clinical and other biological samples such as nasal swabs, blood, buffy coats, dried blood (Hosono *et al.*, 2003; Lasken *et al.*, 2004) cultured cells and tissues (Lasken *et al.,* 2004). It is possible to amplify DNA up to 1000-10000-fold from small amounts of whole blood, dried blood, buccal cells and buffy coats specimen using phi29 DNA polymerase (Hosono *et al.,* 2003). This significant amplification ability of phi29 DNA polymerase can be employed to increase the concentration of template and improve detection of limiting amounts of any nucleic acid of pathogenic microorganism in patient samples when they are below the normal PCR detection limit.

This chapter describes a preliminary study conducted to determine the efficiency of phi29-amplification to amplify different structural conformations of DNA present in limiting concentrations to improve detection of target sequences by PCR. Phi29-amplification was initially used to amplify genomic DNA extracted from bacteriophage lambda (λ) particles. Subsequently, four different structural conformations of DNA — bacteriophage λ DNA (dsDNA 48.0 kb), cDNA prepared from MS2 RNA (sscDNA, 3.6 kb), pUC18 (circular dsDNA, 2.6 kb) and pKEYE (circular dsDNA, 8.6 kb, containing a copy of *Escherichia coli recA* gene) were used in limiting concentrations to determine the efficiency of phi29-amplificaiton on the detection of these targets by PCR.

3.2 Improved detection of bacteriophage λ particles DNA using phi29 DNA polymerase

A stock of bacteriophage lambda (λ) particles was prepared, quantified and serially diluted between 10^{10} pfu ml^{-1} and 10^{1} pfu ml^{-1} (Section 2.5). Bacteriophage λ DNA was extracted from all of these different concentrations and divided into two portions. One aliquot was amplified using the REPLIg kit (QIAGEN, Crawly, UK) and the other aliquot was not amplified. Both of the amplified and non-amplified λ DNA was then used as template for PCR (Section 2.6.7) using λ genome specific primers (Appendix A1). Extracted bacteriophage λ DNA could not be detected below a concentration of 10^{5} pfu ml^{-1} without phi29-amplification (Figure 3.1, L7). However, after phi29-amplification it was possible to detect bacteriophage λ DNA at a lower concentration of 10^{3} pfu ml^{-1} (Figure 3.1, L14). We observed that there was 100-fold improvement in detection of bacteriophage λ DNA after phi29-amplification (Figure 3.1).

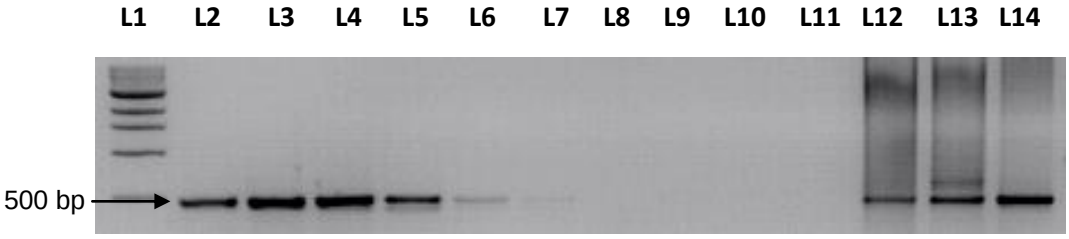

Figure 3.1: PCR detection of extracted lambda DNA before and after phi29 DNA polymerase amplification. Lane: L2-L10: before phi29 DNA polymerase amplification; L12-L14: after phi29 DNA polymerase amplification; L1: 100 bp DNA ladder (NEB, Hitchin, UK); L2: 10^{10}; L3: 10^{9}; L4: 10^{8}; L5: 10^{7}; L6: 10^{6}; L7: 10^{5}; L8: 10^{4}; L9: 10^{3}; L10: 10^{2} pfu ml^{-1}. L11: negative control; L12: 10^{5}; L13: 10^{4}; L14: 10^{3} pfu ml^{-1}.

3.3 Protocol for phi29 amplification of different structural conformations of DNA

A protocol was designed to determine the improvement in detection of four different structures of purified DNA. These four structures of DNA were chosen as representatives of different structural conformations of viral nucleic acids so that this protocol could be evaluated for further applications on clinical samples. Four different types of DNA templates were chosen for this assay, bacteriophage λ DNA (dsDNA 48.0 kb), cDNA prepared from MS2 RNA (sscDNA, 3.6 kb), pUC18 (circular dsDNA, 2.6 kb) and pKEYE (circular dsDNA, 8.6 kb, containing a copy of the *Escherichia coli recA* gene). Each of the four different structural forms of DNA was diluted in 10 mM Tris-HCl (pH 8.0) in different concentrations from a known stock in a 1:10 fold serial dilution to determine the endpoint of detection by PCR. These different concentrations of DNA were amplified using the REPLIg kit (QIAGEN, Crawley, UK) (Section 2.6.8). Amplified DNA was diluted (1:20) in Tris-HCl (pH 8.0) following the protocol provided by the manufacturers (QIAGEN, Crawley, UK) to use as a template for subsequent detection by PCR (Section 2.6.7.2). The DNA concentration was also measured both before and after phi29 DNA polymerase amplification using the Quanti-iT PicoGreen® dsDNA reagent kit (Invitrogen, Paisley, UK) (Section 2.6.2.2).

3.4 Improved detection of different structural conformations of DNA after phi29 DNA polymerase amplification

Different structures of DNA at various concentrations were detected by PCR technique both before and after phi29 amplification to determine any improvement in detection after such amplification. Detection of λ, pKEYE and pUC18 DNA could be improved from 10 to 100-fold but MS2 cDNA could not be amplified significantly after phi29 DNA polymerase amplification (Table 3.1).

Template DNA concentrations were determined both before and after phi29 DNA polymerase amplification using a picogreen dye method (Section 2.6.2). Concentrations of all four structural conformations of DNA were amplified 10^6-10^8-fold after phi29-amplification as determined by the picogreen assay. The amount of amplification was determined by comparing the calculated concentrations of DNA in different dilutions prepared from known stocks with those after phi29-amplification and picogreen assay. It was found that double stranded DNA was amplified more efficiently than the single stranded DNA with phi29 DNA polymerase as determined by picogreen assay (Table 3.1). This assay was repeated three times and similar results were found. The PCR detection was done to determine presence of target sequences and picogreen assay was done for determination of total amount of DNA before and after phi29 amplification. The average value total amount of DNA from three replicate experiments in picogreen assay is shown in table 3.1.

Table 3.1: Detection of different structures of DNA in different concentrations using PCR technique and picogreen dye both before and after phi29 DNA polymerase amplification

DNA (type and size)	Concentrations of DNA	PCR detection of target sequences (copy μl^{-1})					Detection of total amount of DNA, Picogreen assay
		10^3	10^2	10^1	10^0	10^{-1}	
Lambda (dsDNA 48.0 kb)	Before	+	-	-	-	-	Below detection level*
	After	+	+	+	-	-	399.26 µg ml^{-1}
pUC18 (circular dsDNA, 2.6 kb)	Before	+	+	+	-	-	Below detection level*
	After	+	+	+	+	+	197.25 µg ml^{-1}
pKEYE (circular dsDNA, 8.6 kb)	Before	+	-	-	-	-	Below detection level*
	After	+	+	+	-	-	196.05 µg ml^{-1}
MS2 cDNA (sscDNA, 3.6 kb)	Before	+	+	-	-	-	Below detection level*
	After	+	+	-	-	-	186.92 µg ml^{-1}

*Minimum detection level is, 25 nanogram ml^{-1}

3.5 Discussion

We evaluated the application of phi29 DNA polymerase for isothermal amplification of four different structural conformations of DNA from limiting concentrations. These four different structural conformations were selected as representatives of different structural conformations of nucleic acids of viruses so that similar approach can be applied for amplification of genomes of pathogenic viruses from clinical samples. Although it was not possible to cover all variety of virus nucleic acids such as double stranded RNA this study sheds light on how much these selected forms of DNA could be amplified using phi29 DNA polymerase.

It was found that the efficiency of phi29 DNA polymerase mediated amplification varied for the different structural conformations of DNA which could be due to several reasons. Larger DNA fragments (>2.0 kb) are amplified more efficiently than shorter fragments of linear DNA using phi29 DNA polymerase (Bergen *et al.*, 2005; Brukner *et al.*, 2006). On the other hand, small circular structure of DNA (150 bp) could be amplified 10^7-fold using phi29 DNA polymerase in multiply-primed rolling circle amplification (Nelson *et al*., 2002; Brukner *et al.*, 2006) (Section 1.6). The REPLIg kit (QIAGEN, Crawley, UK) was used for amplification of all different types of templates in this study. This kit uses a chemical denaturation step for denaturing template DNA which is critical for binding of primers and efficient amplification of template DNA. It was found that λ DNA, pUC18 and pKEYE plasmid DNA could be amplified efficiently using this system but sscDNA prepared from MS2 could not (Table 3.1). It was also found that the protocol of the REPLIg kit (QIAGEN, Crawley, UK) worked better for linear dsDNA and different sizes of circular dsDNA than the sscDNA in this study. This could be due to the limitation of the REPLIg kit for efficient amplification of ssDNA. Similar amplification of the human genome (Dean *et al*., 2002), plasmid and phage DNA (Dean *et al*., 2001) using phi29 DNA polymerase has been reported by other workers. Improved detection of circular and linear viral dsDNA in clinical samples

by real-time PCR after incorporating a pre-amplification step using phi29 DNA polymerase has recently been reported by other workers (Erlandsson *et al*., 2010).

Single stranded cDNA prepared from MS2 RNA is very labile and degrades quickly which may be one of the reasons for it not being amplified using the whole genome amplification method in the REPLIg kit (Section 2.6.6.2). Shorter lengths of cDNA prepared from the MS2 genomic RNA (3.8 kb) could be another reason for not being amplified after whole genome amplification using phi29 DNA polymerase in the REPLIg kit (Section 2.6.6.2). However, a modified method of ligation-mediated phi29 DNA polymerase amplification was found to be useful for the amplification of RNA of all sizes by other workers (Berthet *et al.*, 2008). Berthet *et al.* (2008) used whole transcriptome amplification (WTA) kit (QIAGEN, Crawley, UK) instead of REPLIg kit (QIAGEN, Crawley, UK) for ligation-mediated amplification of different sizes of virus RNAs. The WTA kit (QIAGEN, Crawley, UK) is relatively expensive compared to any other kits (reagent cost £ 25.0 per sample) and will not be feasible for pre-amplification of nucleic acids before routine laboratory diagnosis by PCR.

The Quanti-iT™ PicoGreen® dsDNA reagent kit (Invitrogen, Paisley, UK) was used to determine the concentration of all four types of templates directly and after phi29 DNA polymerase amplification. Initial concentrations of templates were below the detection level by picogreen assay (Table 3.1 and Section 2.6.2.2) for all four types of templates but their concentrations increased after phi29 DNA polymerase amplification. In this study it was possible to amplify the concentrations of λ, pUC18 and pKEYE DNA up to 399.26 µg ml^{-1}, 197.25 µg ml^{-1} and 196.05 µg ml^{-1} respectively from limiting concentrations (Table 3.1). After comparing the calculated concentration of the diluted templates with those after phi29-amplification it was found that all four structures were amplified 10^6-10^8-fold by picogreen assay. Amplification of single stranded cDNA (prepared from bacteriophage MS2 RNA) showed a concentration of 186.92 µg ml^{-1} of DNA after phi29 amplification in the picogreen assay. However, the target sequence could

not be detected by PCR from this amplified product. This indicates that amplification of single stranded cDNA was actually nonspecific or template independent. It was reported by other workers that the nonspecific or template independent DNA synthesis was completely suppressed when modified primers with two 5'-nitroindole (universal base) were used (Lage *et al*., 2003). In a recent study it was also found that addition of trehalose during phi29-amplification significantly improved the amplification bias and template independent products from as little as 0.5-0.25 nanogram of human genomic DNA or a few cells (Pan *et al*., 2008). Trehalose is a disaccharide that contains glucose molecules bound in an α-1,1 linkage. Trehalose was found to increases the phi29-amplification activity at an elevated temperature of 40° C and helps to maintain activity of the enzyme at 30° C for at least 26 hours (Pan *et al*., 2008). This finding is in concordance with the findings of other workers performing amplification of template DNA from limiting concentrations which could be useful for improving detection of any pathogen present in clinical samples (Brukner *et al.*, 2006; Lasken and Egholm, 2003). Picogreen dye was also used in another study for quantitation of DNA after phi29 DNA polymerase amplification to be used for checkerboard DNA-DNA hybridization (Teles, *et al.*, 2007). It was found that bacterial DNA could be amplified 1000-fold using phi29-amplification as determined by picogreen assay (Teles *et al.*, 2007). In this study, the REPLIg kit (QIAGEN, Crawley, UK) was used for phi29-amplification of template DNA, so there was no scope to change the random primers or add trehalose to the reaction to reduce nonspecific amplification.

Based on the available literature and the findings in this section it is clear that, phi29-amplification may be useful for the amplification of various nucleic acids of pathogenic microorganisms from clinical samples to improve subsequent detection by PCR. Phi29 DNA polymerase amplification could be further improved by adopting modified protocols such as using modified primer having two 5'-nitroindole (universal base) and adding trehalose to the amplification reaction. An alternative cheaper protocol for ligation-mediated phi29-amplification could be

used for the amplification of nucleic acids in order to improve detection of single stranded cDNA. Several modified protocols for ligation-mediated phi29 amplification and a new protocol for RT-Bst amplification have been developed (Chapter 4) to improve detection of single stranded cDNA.

Chapter 4

Development of a novel method — RT-Bst for amplification of viral RNA

4.1 Introduction and aims

The advancements in molecular diagnostic tests have significantly improved our ability to detect pathogenic microorganisms and has expanded our knowledge of the diversity of microorganisms associated with infections (reviewed by Olofsson *et al*., 2011; Delwart, 2007). Crucially, in some instances, pathogens may not be detected due to the lack of sensitivity of the existing methods and/or the presence of previously unrecognised pathogens (Allander *et al*., 2007; Fox, 2007). Unsatisfactory detection of pathogenic viruses of the respiratory tract may also be associated with the lower abundance of virus particles and the poor quality of samples collected from patients. Very low quantities (femtogram levels) of target nucleic acids present in any sample may not be amplified by PCR thereby producing false negative results (Karrer *et al*., 1995; Schloss *et al*., 2003). This limitation of PCR detection could be due to small and random differences in amplification efficiency between individual templates in amplifying nucleic acids. However, cell free nucleic acid amplification techniques using phi29 or the large fragment of *Bst* DNA polymerase (Section 1.6) can be useful for the amplification of large amounts of nucleic acids from a limited amount of nucleic acid or few cells to improve subsequent detection of target sequences by PCR (Dean *et al*., 2001; Lizardi *et al*., 1998). Both phi29 and the large fragment of *Bst* DNA polymerase use random primers and nucleotides to amplify the nucleic acid copy number through multiple displacement amplification (Section 1.6). Random primers bind to the denatured templates and the polymerase extends the primers and replicate the copy of the template DNA. Random primers are simultaneously extended by replicating the template DNA and displacing the 5' end of the other strand from the template producing multiple copies of the starting template. As a result of increasing numbers of priming events the amplified product forms a network of hyper-branched DNA. Phi29 and the large fragment of *Bst* DNA polymerase amplify the template DNA by similar mechanisms at 30° C and 60-65° C, respectively.

PCR-based and non-PCR-based isothermal amplification of target sequences have been found to be useful to improve the sensitivity of PCR detection of nucleic acids from very low concentrations from clinical and laboratory samples (Erlandsson *et al*., 2011 and 2010; Dean *et al*., 2002). Multiple displacement amplification of whole genome using phi29 DNA polymerase was found to be useful for amplification and sequencing of an environmental strain of *Vibrio cholerae* 01 from 10 nanograms of extracted genomic DNA (Pérez Chaparro *et al*., 2011). Circular and linear viral dsDNA could be amplified significantly from clinical samples using phi29 DNA polymerase which improved detection of positive samples normally below the detection limit of specific real time PCR assay (Erlandsson *et al*. 2011).

The large fragment of *Bst* DNA polymerases has been less frequently used for whole genome amplification than phi29 DNA polymerase possibly due to its lower efficiency of amplification of nucleic acids. Lage *et al*. (2003) developed a simple method to generate thousands of copies of a human genome from 500 cells in only a few hours using large fragments of *Bst* DNA polymerase. Other workers also found that multiple displacement amplification (MDA) (Section 1.6) using *Bst* DNA polymerase of intact DNA, such as DNA isolated from fresh or snap-frozen tissue, showed representational distortion of less than three-fold (Lage *et al*., 2003; Dean *et al*., 2002; Rook *et al*., 2004). *Bst* DNA polymerase amplified products were found to be more useful for its fidelity over phi29 DNA polymerase in comparative genomic hybridization array analysis using the human genome (Dean *et al*., 2002; Aviel-Ronen *et al*., 2006).

In a recent study, Erlandsson *et al*., (2011) demonstrated a new diagnostic tool for virus detection by combining microarray detection with phi29-amplification. They showed that this method detects both DNA and RNA viruses and differentiates between different virus subtypes. Human papilloma virus (HPV) specific PCR amplification from low virus titre in clinical samples was found useful for the diagnosis of HPV by subsequent microarray hybridization (Mejlhede *et al*., 2009;

Oh *et al*., 2004). The sensitivity of real-time PCR detection of circular and linear viral dsDNA genomes in clinical samples was increased by Amp-PCR (Erlandsson *et al*., 2010). Amp-PCR technology uses a pre-amplification of whole genome using phi29 DNA polymerase to improve detection of target sequence by specific real-time PCR (Section 1.7).

Nucleic acid sequence based amplification (NASBA) is an *in vitro* isothermal amplification system for RNA (Section 1.5.5). NASBA has been widely used for detection of pathogens in clinical, environmental and food samples (Fu *et al*., 2011; Gracias *et al*., 2007). NASBA has been successfully used by other workers to amplify the copy number of RNA to improve detection of rabies virus (Sugiyama *et al.*, 2003) and rapid confirmation of influenza A H5N1 virus in clinical samples (Moore *et al*., 2010). Real time NASBA using a molecular beacon probe was found to be more sensitive than a cell culture-based method for detection of wide range of influenza A virus subtypes (Moore *et al*., 2004) whereas NASBA alone was found to have sensitivity equivalent to the egg culture method (Shan *et al*., 2003). These culture-based detection methods are still used as the gold standard for virology diagnostics. Signal mediated amplification of RNA technology (SMART) is another method for isothermal amplification of RNA (Wharam *et al*., 2001) (Section 1.6.2; Figure 1.2).

Inefficient template extraction and co-purification of inhibitors during nucleic acid extraction procedures are major hurdles for successful amplification and detection of pathogenic microorganisms by PCR. PCR inhibitors either can bind and interact with a DNA template (Eckhart *et al*., 2000; Bickley *et al*., 1996) or interact with DNA polymerase during primer extension. An application of sequence specific 5'-biotinylated probes for hybridization and concentration of specific sequences using magnetic beads to improve detection of target sequences has been reported by other workers (Li *et al.*, 2011). Magnetic beads have also been used for isolation and concentration of minute quantities of nucleic acids from clinical samples and

improve detection of HPV16 viruses (Peeters, *et al.*, 2011) and hepatitis C viruses (Miyachi *et al.*, 2003).

In the light of previous studies and findings, different protocols were validated in this chapter to enrich low copy number cDNA (prepared from MS2 RNA) to evaluate the performances of the protocols. 5'-biotinylated probes were used to concentrate bacteriophage lambda (λ) and MS2 genome sequences. Ligation-mediated phi29 DNA polymerase amplification was used to enrich the copy number of single stranded cDNA (sscDNA) and double stranded cDNA (dscDNA) prepared from MS2 RNA. CircLigase™ II ssDNA ligase was also used for circularization of single stranded MS2 cDNA to improve amplification of circularized DNA structures using phi29 DNA polymerase. A novel protocol was developed in this study through combining reverse transcription and *Bst* DNA polymerase amplification in a single reaction for synthesis and amplification of cDNA directly from RNA templates in order to improve sensitivity of PCR.

4.2 Concentration of lambda (λ) DNA using a 5'-biotinylated probe in the presence of *Staphylococcus aureus* DNA

Aliquots of lambda (λ) DNA (10^4, 10^2 and 10^1 copy μl^{-1}) were added to a known concentration of *Staphylococcus aureus* DNA (10^4 copy μl^{-1}). Lambda DNA specific 5'-biotinylated and non-biotinylated probes were hybridized with λ DNA present in each sample. Both biotinylated and non-biotinylated probes hybridized to λ DNA were concentrated using streptavidin coated beads (MyOne™ C1 from Invitrogen, Paisley, UK). Briefly, MyOne™ C1 beads were suspended in 2X binding and washing (B&W) buffer to a final concentration of 5 $\mu g\ \mu l^{-1}$. An equal volume of both biotinylated DNA (100 µl) was added in the 2X B&W buffer containing beads and incubated for 15 minutes at room temperature using gentle rotation. The biotinylated DNA coated beads were separated using a magnet for 2-3 minutes,

washed with B&W buffer and resuspended in 50 µl of elution buffer (Tris-HCl, pH 8.5) for further analysis. Non-biotinylated DNA (used as a control) was also concentrated using the same protocol. The efficiency of biotinylated and non-biotinylated probes to enrich λ DNA sequences was compared by PCR (Section 2.6.7) using λ DNA specific primers (Appendix, Table A1). Lambda DNA could not be detected below 10^4 copy μl^{-1} using a non-biotinylated sequence specific probe (Figure 4.1, L2). However, lambda DNA could be detected at a lower concentration (10^2 copy μl^{-1}) after using a 5'-biotinylated sequence specific probe (Figure 4.1, L6). It was found that PCR detection of λ DNA was improved 100-fold after concentrating the DNA using 5'-biotinylated probes (Figure 4.1).

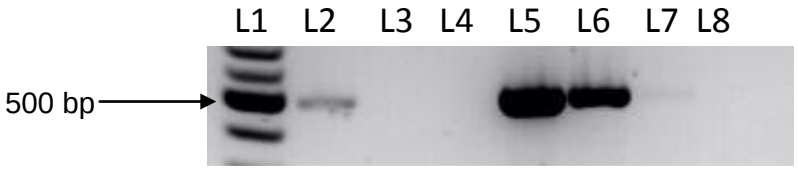

Figure 4.1: Enrichment of λ DNA using 5'-biotinylated probe. L2-L4: after hybridization with non-biotinylated probes; L1: 100 bp DNA ladder (NEB, Hitchin, UK); L5-L7: after hybridization with biotinylated probes; L2 and L5: *Staphylococcus aureus* (Sa) 10^4 copy μl^{-1} + Lambda (λ) 10^4 copy μl^{-1}; L3 and L6: Sa 10^4 copy μl^{-1} + λ 10^2 copy μl^{-1}; L4 and L7: Sa 10^4 copy μl^{-1} + λ 1 copy μl^{-1}; L8: negative control.

Equal amounts of MS2 single stranded cDNA (10^5 copy μl^{-1}) was added to different concentrations of lambda (λ) DNA (0.1 ng μl^{-1}, 0.01 ng μl^{-1}, 1 pg μl^{-1}, 0.1 pg μl^{-1}, 0.01 pg μl^{-1}). MS2 cDNA specific 5'-biotinylated probes were allowed to hybridize with MS2 cDNA. Streptavidin beads, MyOne™ C1, (Invitrogen, Paisley, UK) were used to concentrate MS2 cDNA from these samples and remove excess background λ DNA. Both the target sequences of MS2 and λ DNA were detected by PCR before and after concentrating MS2 cDNA using biotinylated probes and streptavidin beads. It was found that 5'-biotinylated probes (specific for MS2 sequence) were useful to capture MS2 sequences successfully in the presence of

λ DNA at all concentrations of 0.1 ng μl^{-1} to 0.01 pg μl^{-1}. Biotinylated probes (specific for MS2 sequence) were also found to be useful to concentrate MS2 cDNA and remove the λ DNA from the background at a concentration of 0.01 picogram μl^{-1} (Figure 4.2, B5).

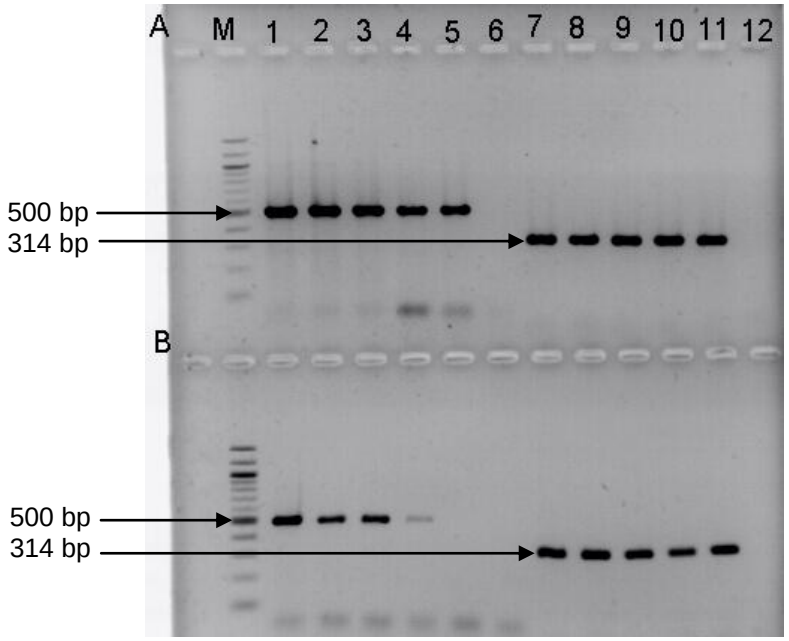

Figure 4.2: PCR detection of MS2 single stranded cDNA and lambda (λ) DNA both before and after MS2 cDNA specific biotinylated probe capture assay. M: 100 bp DNA ladder (NEB, Hitchin, UK); A: before probe hybridization and capture; B: after probe hybridization and capture; A1-5 & B1-5: PCR detection of λ DNA; A7-11 & B7-11: PCR detection of MS2 sscDNA; 1 and 7: λ (0.1 ng μl^{-1}) + MS2 (10^5 copy μl^{-1}); 2 and 8: λ (0.01 ng μl^{-1}) + MS2 (10^5 copy μl^{-1}); 3 and 9: λ (1 pg μl^{-1}) + MS2 (10^5 copy μl^{-1}); 4 and 10: λ (0.1 pg μl^{-1}) + MS2 (10^5 copy μl^{-1}); 5 and 11, λ (0.01 pg μl^{-1}) + MS2 (10^5 copy μl^{-1}); 6 and 12: negative control; Amplicon sizes; λ DNA: 500 bp; MS2: 314 bp.

4.3 Protocols for ligation-mediated phi29-amplification to enrich the target sequences

In the previous chapter (Chapter 3), it was found that double stranded DNA (dsDNA), either linear or circular forms could be amplified efficiently using phi29 DNA polymerase. This was not the case for single stranded cDNA (sscDNA). One of the reasons could be that the sizes of cDNA converted from MS2 RNA were relatively small; the genome of MS2 RNA is only 3.8 kb. Moreover, sscDNA is more labile and degrades more easily than dsDNA. It was also found that the larger molecules of linear DNA (>2 kb) are amplified more easily than the smaller ones using phi29 DNA polymerase (Dean *et al.*, 2001; Brukner *et al.*, 2006). This led to the suggestion that different protocols will be necessary for the successful amplification of sscDNA such as ligation of sscDNA creating concatemers to increase their size before phi29 DNA polymerase amplification (Berthet *et al.*, 2008). Phi29 DNA polymerase binds to any template DNA and replicates the DNA up to 70 kb without dissociating from the DNA strand. Therefore a larger molecular weight DNA molecule will be amplified more efficiently than lower molecular weight one.

4.3.1 T4 RNA ligase-mediated phi29-amplification of MS2 sscDNA

T4 RNA ligase assay was performed in the presence and absence of carrier RNA to determine the effect of carrier RNA in ligation-mediated phi29-amplification. The carrier RNA was used from the QIAamp RNA extraction Mini Kit (QIAGEN, Crawley, UK) in order to protect the target RNA from degradation and improve the yield during spin column extraction. One assay was set up adding MS2 RNA (10^7 copy μl^{-1}) to carrier RNA (50 ng μl^{-1}) and another adding MS2 RNA (10^7 copy μl^{-1}) only without carrier RNA. Both reactions were further diluted to 10^3 copy μl^{-1} and reverse transcribed to sscDNA using 5'-phosphorylated random hexamers (Appendix, Table A1) according to the protocol described in section 2.6.4. The

diluted cDNA (10^7 to 10^3 copy μl^{-1}) was ligated using T4 RNA ligase according to table 4.1.

Table 4.1: Ligation mix for sscDNA using T4 RNA ligase

Reaction component	**Reaction volume**
Linear sscDNA	2-20 ng or 12.0 µl
10X reaction buffer	2.0 µl
T4 RNA ligase (Fermentas, York, UK)	10 U or 1.0 µl
ATP 10 mM	1.0 µl
BSA 1 mg ml^{-1}	2.0 µl
Nuclease free water	2.0 µl
Total volume	20.0 µl
Incubation	37 ºC for 30 min
Inactivation	70 ºC for 10 min

As a result of this ligation of the 5'-phosphorylated cDNA with T4 RNA ligase, single stranded cDNA (sscDNA) is predicted to form concatemers of sscDNA in linear or circular structures (Figure 4.3).

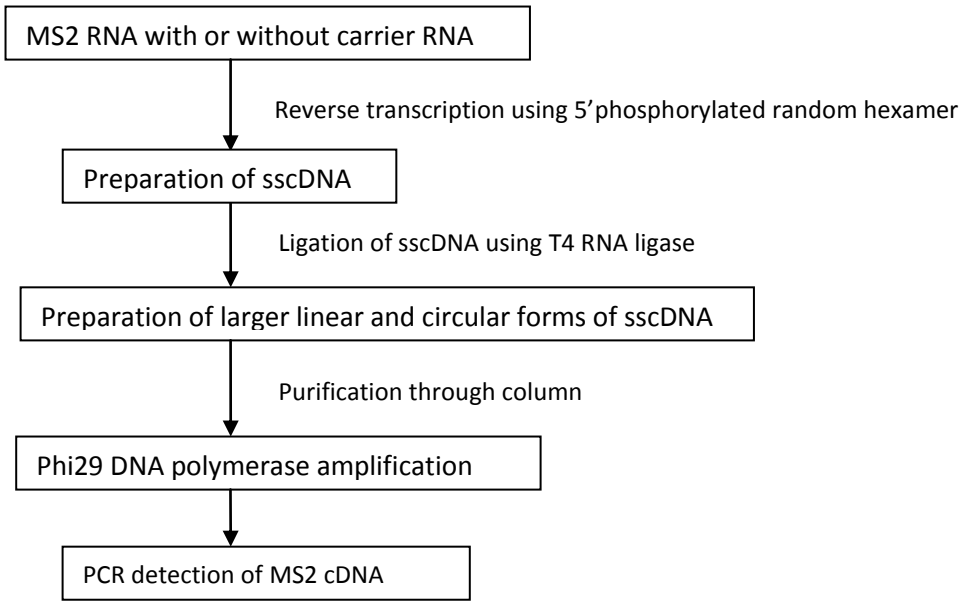

Figure 4.3: Flowchart showing the protocol for T4 RNA ligase-mediated phi29-amplification

Ligated products were purified through DNA purification columns and immediately amplified using the REPLIg kit (QIAGEN, Crawley, UK) for subsequent detection by PCR. MS2 sequence could be detected at a concentration of 10^4 copy μl^{-1} (Figure 4.4, A4) after phi29-amplification without a ligation step whereas the same sequence could be detected at a lower concentration of 10^3 copy μl^{-1} (Figure 4.4, B5) after including a ligation step (in the presence of carrier RNA). This assay demonstrated that PCR detection of MS2 sequence was improved 10-fold after ligation-mediated phi29-amplification in the presence of carrier RNA (Figure 4.4). On the other hand, MS2 sequence could be detected at a concentration of 10^3 copy μl^{-1} (Figure 4.4, A11) without ligation and phi29-amplification in the absence of carrier RNA. The sensitivity of PCR detection of MS2 sequence was observed to be lower and could only detect MS2 sequence at a higher concentration (10^4 copy μl^{-1}) after ligation-mediated phi29-amplification in the absence of carrier RNA (Figure 4.4, B10). This confirmed that ligation-mediated phi29-amplification was

useful to improve detection (10-fold) of target sequence in the presence of carrier RNA whereas the same assay was less sensitive (10-times) in the absence of carrier RNA. There were some non-specific bands observed after PCR amplification (Figure 4.4) of the ligated and phi29-amplified products which could be due to the formation of concatemers during this ligation assay.

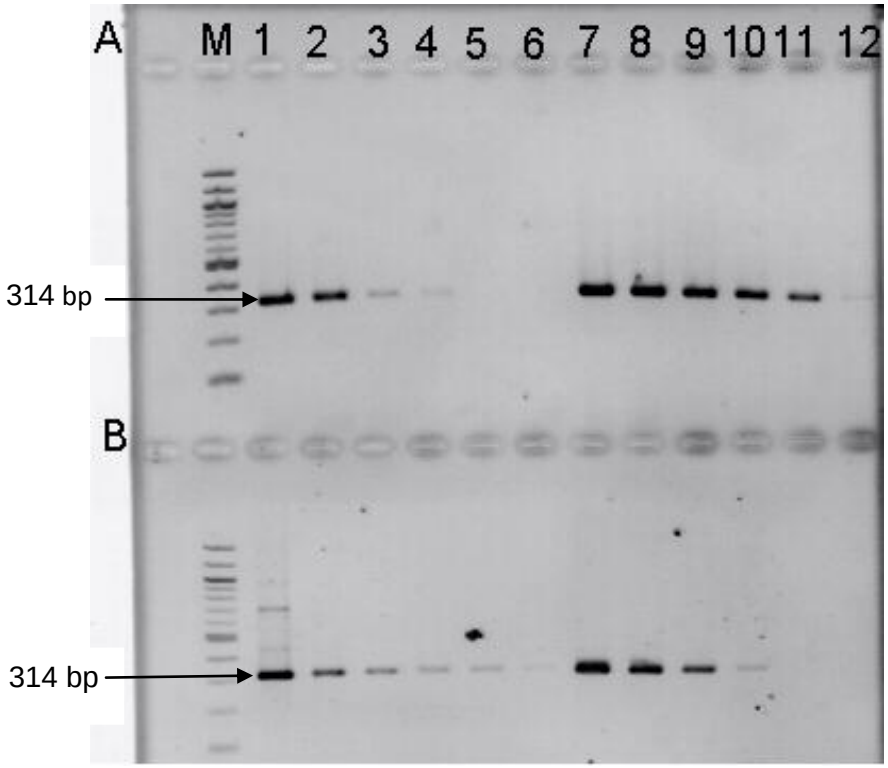

Figure 4.4: T4 RNA ligase-mediated phi29-amplification in the presence or absence of total RNA. A 1-12: before phi29-amplification; B1-12: after phi29-amplification using REPLIg kit (QIAGEN, Crawley, UK). M: 100 bp DNA ladder (NEB, Hitchin, UK); A 1-5 and B 1-5: MS2 RNA with carrier RNA (50 ng μl^{-1}); A 7-11 and B 7-11: MS2 RNA only as control. A1, A7, B1, B7: 10^7 copy μl^{-1}; A2, A8, B2, B8: 10^6 copy μl^{-1}; A3, A9, B3, B9: 10^5 copy μl^{-1}; A4, A10, B4, B10: 10^4 copy μl^{-1}; A5, A11, B5, B11: 10^3 copy μl^{-1} MS2 RNA; A6, A12, B6, B12: negative control; MS2 amplicon size: 314 bp.

4.3.2 T4 DNA ligase-mediated phi29-amplification of double stranded cDNA generated from MS2 RNA

4.3.2.1 Preparation of blunt end double stranded cDNA

Single stranded cDNA was prepared from MS2 RNA (10^8 copy μl^{-1}) according to the protocol described in section 2.6.4. Second strand synthesis was performed using DNA polymerase I according to the method described in Table 4.2.

Table 4.2: Reaction mix for second strand synthesis from sscDNA

Reaction component	Reaction volume
From first strand synthesis reaction	20.0 µl
10X reaction buffer for DNA Polymerase I	8.0 µl
RNase H, *E coli* (Fermentas, York, UK)	0.2 µl
DNA Polymerase I, *E. coli* (Fermentas, York, UK)	3 µl (30 U)
Water, nuclease-free	Up to 100 µl
Total volume	100 µl

The reaction mix (Table 4.2) was vortexed gently, centrifuged briefly and incubated at 15º C for 2 hours. 2.5 µl (12.5 U) of T4 DNA polymerase was added to the reaction and incubated at 15º C for 5 minutes for generating blunt end double stranded cDNA. This reaction was terminated by adding 5 µl of 0.5 M EDTA, pH 8.0. Prepared double stranded cDNA was purified through a DNA purification column (QIAGEN, Crawley, UK) and used in T4 DNA ligase reactions.

4.3.2.2 Ligation of double stranded cDNA produced from MS2 RNA

Blunted double stranded cDNA was ligated using T4 DNA ligase according to the protocol in Table 4.3 in the presence of polyethylene glycol 4000 at a final

concentration of 5% (w/v). Such a ligation reaction using T4 DNA ligase will generate two different types of products, both linear and circular forms which may be concatemers of double stranded cDNA derived from MS2 RNA.

Table 4.3: Ligation of double stranded cDNA using T4 DNA ligase

Reaction component	Reaction volume
Linear sscDNA	2.0-20.0 ng or 12.0 µl
10X reaction buffer	2.0 µl
T4 DNA ligase (Fermentas, York, UK)	2.0 U or 0.2 µl
50% (w/v) PEG 4000	2.0 µl
Nuclease free water	3.8 µl
Total volume	20.0 µl
Incubation	22 ºC for 1 h
Inactivation	65º C for 10 min or 70º C for 5 min

4.3.2.3 T4 DNA ligase-mediated phi29-amplification

Double stranded blunt end MS2 cDNA was prepared from a stock of MS2 RNA (10^8 copy μl^{-1}) and divided into two fractions. Complementary DNA (cDNA) in one portion was ligated with T4 DNA ligase and the other was untreated. Both of these ligated and non-ligated double stranded cDNAs were diluted to 10^6-10^3 copy μl^{-1} and detected by PCR (Section 2.6.7.2). Both of these sets of dilutions prepared from ligated and non-ligated double stranded cDNA were further amplified using the REPLIg kit (QIAGEN, Crawley, UK) and subsequently detected by PCR (Figure 4.5). It was found that phi29 DNA polymerase improved the detection of the ligated products more than that of the non-ligated products. But it was not clear from Figure 4.5 (B1-4) how much amplification was achieved after such ligation

and amplification. So this assay was repeated for one of the dilutions (10^8 copy μl^{-1}), to determine the magnitude of amplification through ligation-mediated phi29-amplification (Figure 4.6).

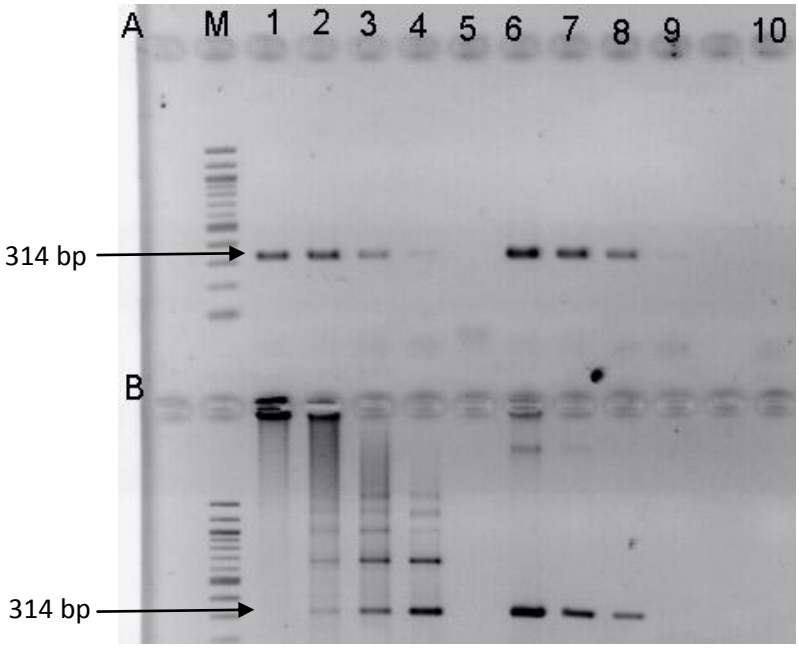

Figure 4.5: Effect of T4 DNA ligase-mediated phi29-amplification to increase copy number of double stranded MS2 cDNA at different concentrations. A1-10: before phi29-amplification; B1-10: after phi29-amplification using REPLIg kit (QIAGEN, Crawley, UK); M: 100 bp DNA ladder (NEB, Hitchin, UK); A1-4 and B1-4: double stranded cDNA ligated with T4 DNA ligase; A 6-9 and B 6-9: double stranded cDNA was not ligated with T4 DNA ligase; A1 and A6: 10^6 copy μl^{-1}; A2 and A7: 10^5 copy μl^{-1}; A3 and A8: 10^4 copy μl^{-1}; A4 and A9: 10^3 copy μl^{-1} of MS2 RNA; A5 and A10: negative control; B1 and B6: 10^6 copy μl^{-1}; B2 and B7: 10^5 copy μl^{-1}; B3 and B8: 10^4 copy μl^{-1}; B4 and B9: 10^3 copy μl^{-1}; B5 and B10: negative control; MS2 amplicon size: 314 bp.

Another assay was set up to ligate double stranded cDNA (10^8 copy μl^{-1}) which was subsequently diluted to 10^5 copy μl^{-1}. In a control tube double stranded cDNA (10^8 copy μl^{-1}) was not ligated by T4 DNA ligase but diluted to 10^5 copy μl^{-1}. These ligated and non-ligated products were amplified by REPLIg kit (QIAGEN, Crawley, UK) for subsequent detection by PCR (Figure 4.6). Ligated double stranded cDNA (prepared form MS2 RNA) was amplified $>10^5$ times compared to the non-ligated double stranded cDNA (Figure 4.6, B). This result showed that ligation of double stranded cDNA with T4 DNA ligase before phi29-amplification was useful for increasing the template copy number to improve detection of the target sequence (MS2 cDNA). Multiple bands appeared after PCR amplification of ligated products (Figure 4.6, A1-7) which could be due to concatemers formation after ligation using T4 DNA ligase. A faint band (314 bp) was also observed in Figure 4.6 (B6), which was possibly due to carryover contamination of template during PCR amplification. All of the assays in this section were performed in triplicate to determine the reproducibility of the results.

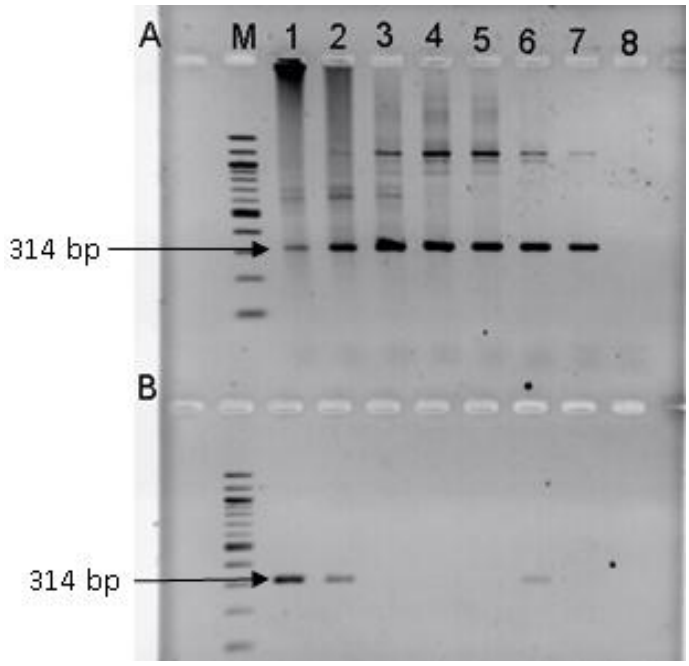

Figure 4.6: T4 DNA ligase-mediated phi29-amplification to enrich copy number of double stranded cDNA (prepared from MS2). A 1-8: after ligation and phi29-amplification; B 1-8: without ligation and phi29-amplification; (1) 1:20; (2) 1:200; (3) 1:2000; (4) 1:20000; (5) 1:200000; (6) 1:2000000; (7) 1:20000000; (8) negative control; M: 100 bp DNA ladder (NEB, Hitchin, UK).

4.3.3 CircLigase™-mediated phi29-amplification of single stranded MS2 cDNA

Circular structures of DNA can be amplified efficiently using phi29 DNA polymerase which is not possible for low molecular weight linear DNA (Liu *et al*., 1996; Dean *et al.*, 2001; Gadkar *et al*., 2011). Phi29 DNA polymerase synthesizes up to 70 kb of DNA once it binds to any template DNA. So, when this enzyme binds to small linear DNA, it replicates a copy of the DNA and dissociates from the template. In contrast, small circular DNA can be amplified multiple times once phi29 DNA polymerase binds to it. Such amplification of circular DNA will significantly improve detection of target sequence. CircLigase™ II ssDNA

(Epicentre Biotechnology, Cambridge, UK) ligase was used in this study for circularization of single stranded MS2 cDNA and to determine any improvement in amplification using REPLIg kit (QIAGEN, Crawley, UK). CircLigase™ II ssDNA ligase is a thermostable ATP dependent enzyme that preferably catalyses intramolecular ligation of ssDNA. This enzyme was isolated from a thermophilic bacteriophage TS2126 (Blondal *et al*., 2005). This enzyme efficiently ligates the 5'-phosphate and 3'-hydroxyl group of ssDNA (>15 bases) at 60-65° C.

Single stranded cDNA was prepared from MS2 RNA stock (10^8 copy μl^{-1}) (Section 2.6.4) using 5'-phosphorylated random hexamers (Appendix, Table A1) to generate 5'-phosphorylated cDNA. This 5'-phosphorylated cDNA was divided into two fractions. One fraction was treated with CircLigase™ II ssDNA ligase and the other fraction was untreated to compare the effect of CircLigase™ II ssDNA ligase-mediated phi29-amplification. Circular structures of cDNA were prepared from linear structures of 5'-phosphorylated cDNA using CircLigase™ II ssDNA ligase according to the protocol in Table 4.4.

Both ligated (circularized) and non-ligated (linear sscDNA) were purified through DNA purification columns and amplified with the REPLIg kit (QIAGEN, Crawley, UK). Both of these amplicons were diluted in 1:10 dilution series to 1:($2x10^{-7}$) dilutions to be used as templates for detection of MS2 sequence. PCR detection of MS2 sequence after phi29-amplification was compared with that without phi29 DNA polymerase amplification. CircLigase™ II ssDNA ligase-mediated phi29-amplification was found to enhance PCR detection of MS2 sequence >10^7-fold compared to the non-ligated sscDNA (Figure 4.7). However, nonspecific bands (Figure 4.7, A1-3) were observed after PCR amplification which is possibly due to the synthesis of concatemers of templates after ligation mediated phi29-amplification. All of the assays in this section was carried out in triplicate and found similar results.

Table 4.4: Reaction mix for CircLigase™ II ss DNA ligase

Reaction component	**Reaction volume**
sscDNA	10 pM or 12.0 µl
CircLigase II 10X reaction buffer	2.0 µl
Magnesium chloride, $MgCl_2$ (50 mM)	1.0 µl
5M Betain (optional)	4.0 µl
CircLigase™ II ss DNA ligase (100 U μl^{-1}) (Epicentre Biotechnology, Cambridge, UK)	1.0 µl
Total volume	20.0 µl
Incubation	60° C for 1 hour
Inactivation	80° C for 10 minutes

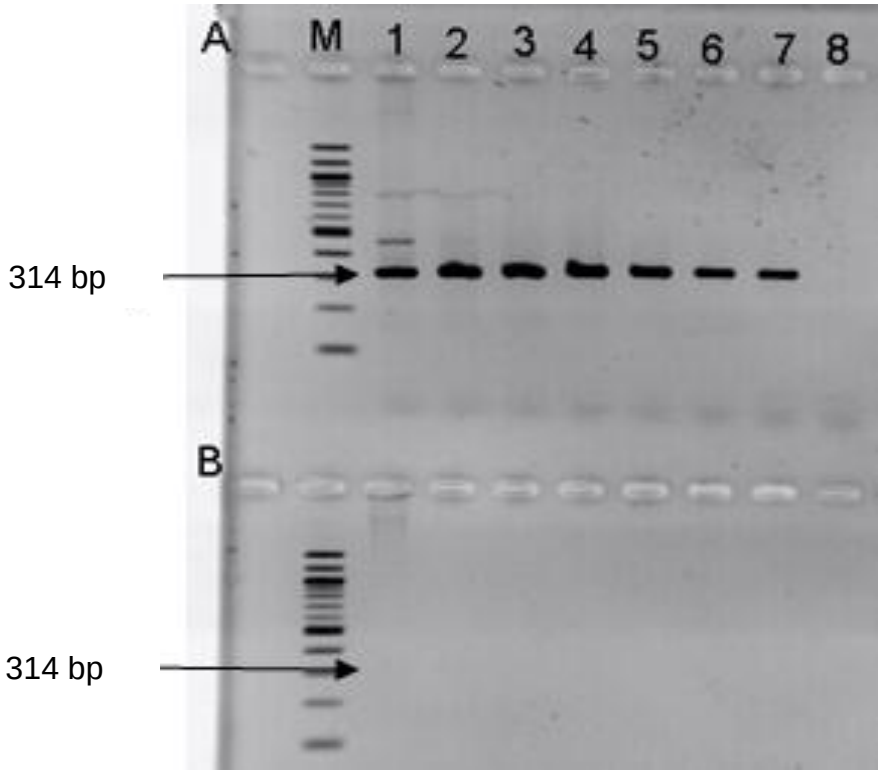

Figure 4.7: Effect of CircLigase™ II ssDNA ligase-mediated phi29-amplification to improve detection MS2 sequence by PCR. M: 100 bp DNA ladder (NEB, Hitchin, UK); A 1-8: after ligation and phi29 DNA polymerase amplification; B 1-8: without ligation but after phi29 DNA polymerase amplification; (1) 1:20; (2) 1:200; (3) 1:2000; (4) 1:20000; (5) 1:200000; (6) 1:2000000; (7) 1:20000000; (8) negative control; MS2 amplicon size: 314 bp.

4.4 Development of a novel protocol — RT-Bst for reverse transcription of RNA and amplification of cDNA

RT-Bst is a novel protocol for reverse transcription and *Bst* polymerase amplification in a one tube reaction (Figure 4.8). This will significantly amplify the cDNA copy number in the RT-Bst reaction compared to that of reverse transcription alone.

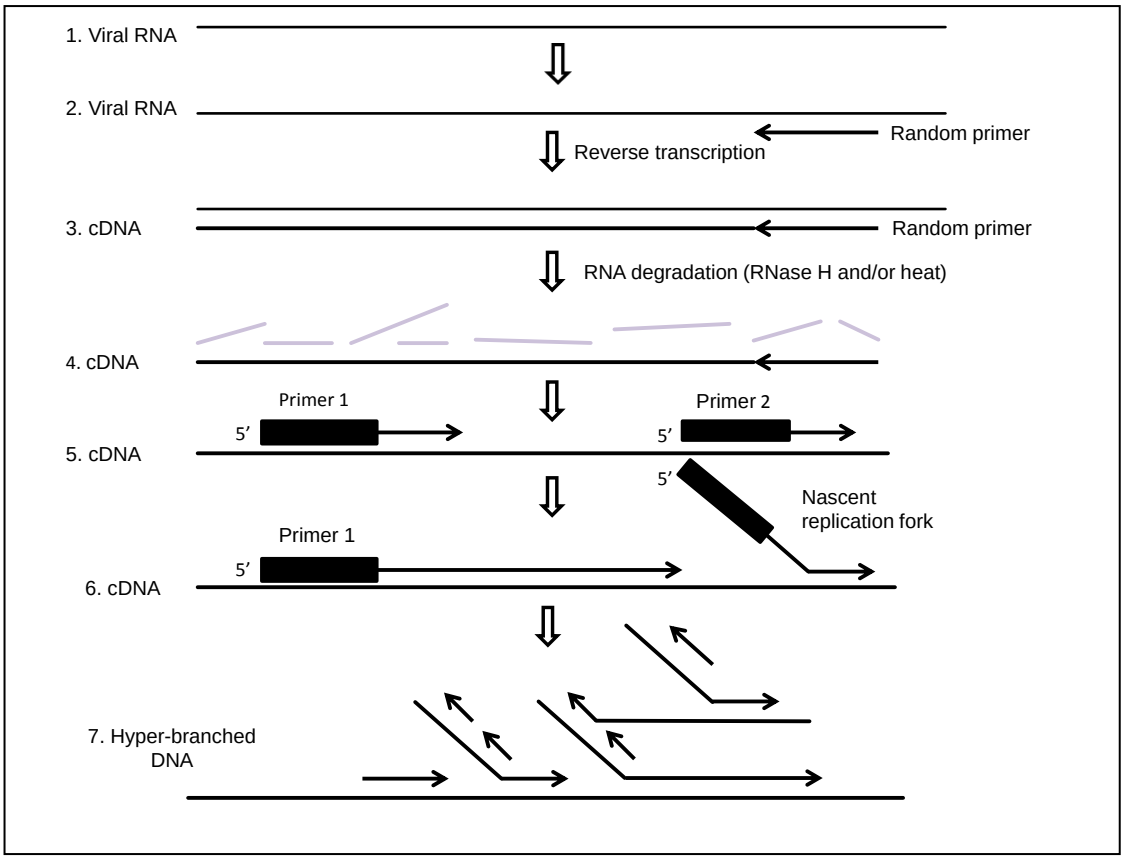

Figure 4.8: Flow chart of RT-Bst amplification. (1) viral RNA; (2) random primers bound to viral RNA; (3) synthesis of cDNA; (4) RNA is degraded due to RNase H activity or heat; (5) random primers bound to sscDNA; (6) extension of random primers; (7) synthesis of many copies of sscDNA (hyper-branched).

An assay was set up to compare the performance of RT-Bst for amplification of known concentrations of MS2 RNA. Reverse transcription is an inefficient process that converts 30-40% of input RNA to cDNA in the reaction (Stangegaard *et al*., 2006). PCR detection of RNA sequence appears to be less sensitive due to the lower efficiency of the reverse transcription especially when the initial RNA is present in low concentration. A novel protocol was designed combining reverse transcription and *Bst* DNA polymerase amplification (RT-Bst) together to boost the synthesis of cDNA from RNA templates. The RT-Bst reaction was prepared according to table 4.5. RNase inhibitor was obtained from Fermentas, York, UK; Moloney Murine Leukemia Virus (MMuLV) and Avian Myeloblastosis Virus (AMV) reverse transcriptase**s** were obtained from NEB, Hitchin, UK; *Bst* DNA polymerase was obtained from Cambridge Biosciences, Cambridge, UK.

Table 4.5: RT-Bst reaction for preparation and amplification of cDNA

Reaction component	**Reaction volume**
Nuclease free water	4.23 µl
10X RT buffer	1.0 µl
N15 random primer (10 µM)	1.0 µl
dNTP (10 mM each)	0.5 µl
RNase inhibitor (40.0 U μl^{-1})	0.25 µl
MMuLV (200 U μl^{-1}) or AMV (10.0 U μl^{-1}) reverse transcriptase	0.125 µl
Bst DNA polymerase (8.0 U μl^{-1})	0.4 µl
Template RNA	2.5 µl
Total	10.0 µl

Incubation conditions for RT-Bst amplification: RT-Bst amplification was performed in a thermocycler in three steps for reverse transcription of RNA

templates and subsequent amplification of cDNA. The RT-Bst reaction was incubated at 25º C for 10 minutes to allow hybridization of random primers, 42º C for 30 minutes for reverse transcription and 60º C for 1 hour for *Bst* DNA polymerase amplification. The reaction was inactivated by heating at 85º C for 5 minutes.

After RT-Bst amplification concentrations of cDNA templates are amplified to a higher level which enhances sensitivity of detection by PCR. 1 µl of RT-Bst amplified cDNA was added in 20 µl HotStarTaq™ PCR reaction as described in section 2.6.7 for detection of MS2 virus sequences.

Two different concentrations of MS2 RNA 8 ng μl^{-1} and 40 ng μl^{-1} were reverse transcribed using the QuantiTect™ Reverse Transcription kit (QIAGEN, Crawley, UK). The same concentrations of RNA were converted to cDNA using RT-Bst reaction using MMuLV and AMV reverse transcriptase as mentioned in Table 4.5. The performances of the three types of reverse transcription reactions were compared qualitatively from the intensity of PCR bands (Figure 4.9). This assay was repeated in triplicate and similar results were observed. The efficiency of RT-Bst amplification is further investigated in Section 4.4.1 and 4.4.2 for amplification and detection of different sites of hRSV genome.

RT-Bst amplification using MMuLV reverse transcriptase was found to be better than QuantiTect™ reverse transcription kit (QIAGEN, Crawley, UK) and AMV reverse transcriptase (NEB, Hitchin, UK) in this study. However, a quantitative assay will be required for further validation of this novel method.

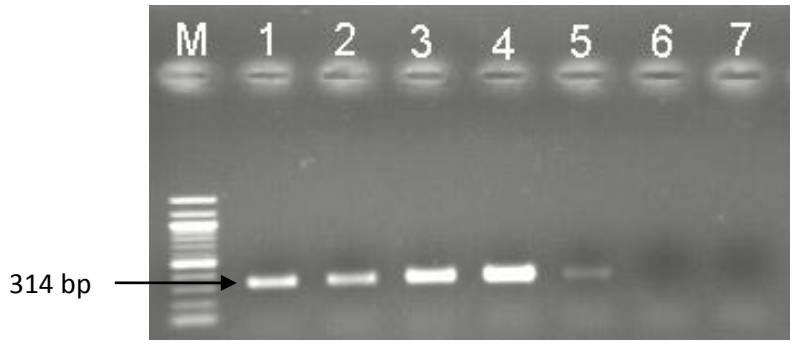

Figure 4.9: RT-Bst amplification using different types of reverse transcriptases. M: 100 bp DNA ladder (Fermentas, York, UK); 1 and 2: reverse transcription using QuantiTect™ reverse transcription kit (QIAGEN, Crawley, UK); 3 and 4: RT-Bst amplification using MMuLV reverse transcriptase (NEB, Hitchin, UK); 5 and 6: RT-Bst using AMV reverse transcriptase (NEB, Hitchin, UK); 7: negative control; 1, 3 and 5: MS2 RNA (8 ng μl^{-1}); 2, 4 and 6: MS2 RNA (40 ng μl^{-1}); MS2 amplicon size: 314 bp.

4.4.1 Relative efficiency the QuantiTect™ reverse transcription kit and the RT-Bst reaction for reverse transcription of the hRSV genome

It appears from the results in section 4.4 that viral RNA could be reverse transcribed and cDNA enriched efficiently using a RT-Bst amplification technique. However, it was not clear from this work whether it amplified whole genomic RNA or a particular portion of genomic RNA compared to the QuantiTect™ reverse transcription kit (QIAGEN, Crawley, UK). Eight pairs of primers were designed to span the entire genome of hRSV (Gene bank accession number AF013254) using the primer BLAST site from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). These eight pairs of primers were designed to determine representative amplification of the entire genome of hRSV after RT-Bst amplification by PCR (Table 4.6, Figure 4.10). Genomic RNA of hRSV was extracted from tissue culture

medium using the QIAamp™ RNA mini kit (QIAGEN, Crawley, UK) (Section 2.6.3). Complementary DNA (cDNA) was prepared from 2.5 µl of extracted RNA using both the QuantiTect™ reverse transcription kit (Section 2.6.4) and the RT-Bst protocol using Maxima reverse transcriptase (Fermentas, York, UK) (Section 2.6.6). 1 µl of cDNA after reverse transcription and RT-Bst amplification was used in 20 µl of PCR reaction for amplification and detection of 8 target sites of hRSV genome. Eight pairs of primers with their corresponding positions in the hRSV genome and amplicon sizes are shown in table 4.6 and figure 4.10.

Table 4.6: Primers used to represent the entire genome of hRSV

Primer sequences (5'-3')	Primer number and orientation	Primer position	Amplicon size
5'-ACGGACATGAGACCCCTGTCGA-3'	Primer-1-F[1]	671-692	532 bp
5'-TGCTGGATGACAGCAGCTGATCC-3'	Primer-1-R[2]	1181-1203	
5'-AGGACCCACTTCAGCTCGCGAT-3'	Primer-2-F	2818-2839	363 bp
5'-GTTGGTGGTTCCGCTGACGGA-3'	Primer-2-R	3161-3181	
5'-TGGTGTCGCAAAACCACGCCA-3'	Primer-3-F	4576-4596	149 bp
5'-AGTCCTGGCAGTGCGTTGATTCT-3'	Primer-3-R	4703-4725	
5'-CCACCCCCGAAAACACACCCAA-3'	Primer-4-F	5495-5516	498 bp
5'-CTCTTCTGGCCCGGTTGTTGGC-3'	Primer-4-R	5972-5993	
5'-AGCTGGGGCAAATATGTCGCGA-3'	Primer-5-F	7605-7626	214 bp
5'-TGTCCAGTTCAGCAGCTCCACT-3'	Primer-5-R	7798-7819	
5'-AGCTTAGGGCTGAGATGTGGATTCA-3'	Primer-6-F	9400-9424	565 bp
5'-GCATTCCTTAAAGTGGGCCATCTGT-3'	Primer-6-R	9941-9965	
5'-GGGTGGTATTGAGGGCTGGTGTC-3'	Primer-7-F	10830-10852	920 bp
5'-GACCCTAACGCCTGTGGATCCCT-3'	Primer-7-R	11683-11705	
5'-GCCATGGGTAGGTTCATCTACGCA-3'	Primer-8-F	12288-12311	422 bp
5'-TGTTCCACAACCGACATCAGGCT-3'	Primer-8-R	12688-12710	

[1] Forward, [2] Reverse

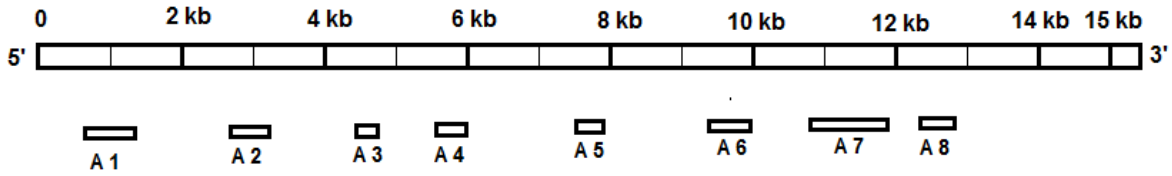

Figure 4.10: Schematic diagram of hRSV genomic RNA (linear 15225 bp). A1 to A8 shows amplicons corresponding to the 8 pair of primers in table 4.6.

QuantiTect™ reverse transcription kit (Figure 4.11). This assay in this section was repeated in triplicate and similar result was found.

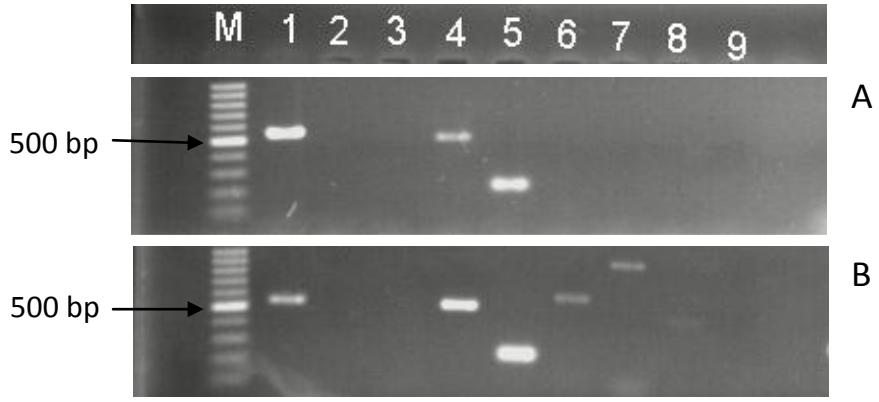

Figure 4.11: Relative efficiency of reverse transcription of the hRSV genome using the QuantiTect™ kit and the RT-Bst amplification. A: Reverse transcription of hRSV RNA using QuantiTect™ reverse transcription kit (QIAGEN, Crawley, UK); B: RT-Bst amplification using Maxima reverse transcriptase (Fermentas, York, UK); M: 100 bp DNA ladder (Fermentas, York, UK); 1-8: PCR detection for 8 different sites of hRSV genome (Table 4.6); 9: negative control.

4.4.2 Effect of MMuLV and Maxima™ reverse transcriptases for RT-Bst amplification of the entire hRSV genome

Based on the assays in sections 4.4 and 4.4.1 it was found that RT-Bst amplification was more efficient for the preparation of cDNA from viral RNA for subsequent detection by PCR. Two enzymes, MMuLV (NEB, Hitchin, UK) and thermostable form of MMuLV, Maxima™ reverse transcriptase (Fermentas, York, UK) were used in RT-Bst amplification to compare their efficiencies for amplification of hRSV genome (Figure 4.12). Reverse transcriptions using MMuLV and Maxima™ reverse transcriptase were carried out according to the protocol described in Section 4.4 and 2.6.6**,** respectively, using 2.5 µl of template RNA.

Uniform and representative amplification of the whole genome was determined by PCR technique using 8 pairs of designed primers spanned throughout the entire hRVS genome (Table 4.6). According to the company information (Fermentas, York, UK) Maxima™ reverse transcriptase is a thermostable form of MMuLV reverse transcriptase which contains RNA dependent and DNA dependent DNA polymerase activity along with RNase H activity. The Maxima™ reverse transcriptase (engineered enzyme) was found to improve the thermostability, robustness, and rate of synthesis of wild type MMuLV reverse transcriptase. Due to the thermostable nature the Maxima™ reverse transcriptase can remain fully active during the entire reverse transcription reaction. The Maxima™ reverse transcriptase can act on a range of input total RNA and can transcribe the secondary structures more efficiently at an elevated temperature (optimum temperature 50-65º C) than the wild type MMuLV reverse transcriptase (optimum temperature 37º C).

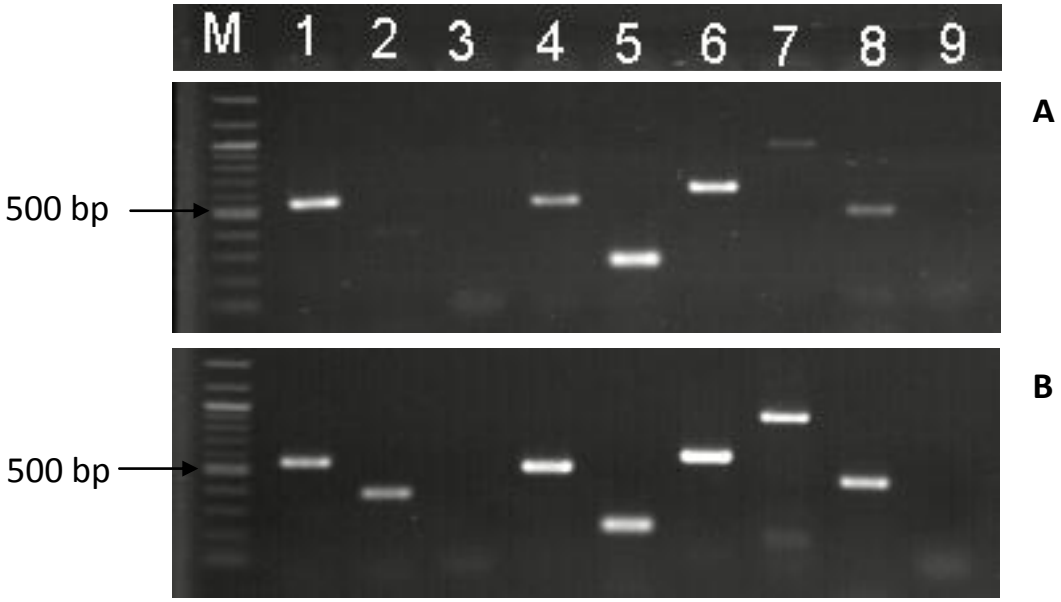

Figure 4.12: Effect MMuLV and Maxima™ reverse transcriptase for RT-Bst amplification of the entire hRSV genome. A: RT-Bst amplification using MMuLV reverse transcriptase (NEB, Hitchin, UK); B: RT-Bst amplification using maxima reverse transcriptase (Fermentas, York, UK); M: 100 bp DNA ladder (Fermentas, York, UK); 1-8: PCR detection for sites 1-8 of hRSV genome (Table 4.6); 9: negative control.

It was found that Maxima™ performed better than the MMuLV reverse transcriptase and showed more intense bands on agarose gels following PCR amplification in Lane 2, 4, 6, 7 and 8 in Figure 4.12. This assay was repeated in triplicate with similar results to verify the reproducibility of this result. Although we determined the efficiency of RT-Bst amplification of the hRSV genome using a qualitative PCR assay a quantitative PCR assay could be more useful to determine this amplification efficiency.

4.4.3 RT-Bst amplification of the entire hRSV genome using random and specific primers

An assay was set up to determine how efficiently RT-Bst could amplify the entire genome of hRSV using random and specific primers. It is understood from the work described in section 4.4.1 that random primers can initiate sequence independent amplification of all different types of nucleic acids templates present in the reaction. Sequence specific primers (8 pairs) were used in this assay for specific amplification of the entire genome of hRSV. Genomic RNA extracted from hRSV (tissue culture media) was reverse transcribed separately using the RT-Bst protocol (Section 2.6.6) using both pentadecamer primer (N15) (Appendix, Table A1) and 8 pairs of hRSV specific primers (Table 4.6) and the RevertAid™ Premium reverse transcriptase (Section 4.4). According to the company information (Fermentas, York, UK) the RevertAid™ Premium reverse transcriptase is a thermostable form of MMuLV reverse transcriptase similar to the Maxima™ reverse transcriptase as discussed before (Section 4.4.2). However, the RNase H activity of the MMuLV reverse transcriptase is eliminated by incorporating a point mutation in the RNase H domain of the MMuLV reverse transcriptase. Mutation in the RNase activity will ensure high yields of full length of cDNA (up to 20 kb). For 8 pairs of primers, equimolar concentrations (0.5 µM) of both forward and reverse primers were added instead of random primers to set up the RT-Bst reaction. MS2 RNA was added as carrier RNA (Section 2.6.3) instead of total RNA to protect hRSV RNA from degradation. This excess amount of MS2 RNA in the reaction will also help to determine the efficiency RT-Bst amplification using nonspecific and specific primers.

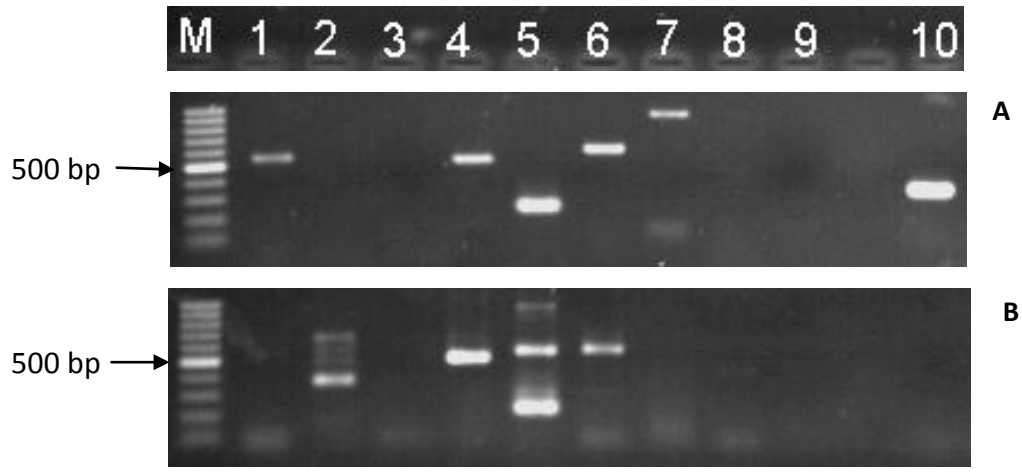

Figure 4.13: RT-Bst amplification of the entire hRSV genome using random and multiplex (sequence specific) primers. A: RT-Bst amplification using N15 random primers and the RevertAid™ Premium reverse transcriptase (Fermentas, York, UK); B: RT-Bst amplification using 8 pairs of hRSV genome specific primers and RevertAid™ Premium reverse transcriptase (Fermentas, York, UK); M: 100 bp DNA ladder (Fermentas, York, UK); 1-8: PCR detection for 8 different fragments of hRSV (Table 4.6); 9: negative control; 10: MS2 RNA.

The RT-Bst amplification using both the random and genome specific primers were successful for amplification of the central portion of the hRSV genomic RNA but no detectable amplification was observed for the terminal sequences of the hRSV genome when sequence specific primers were used for RT-Bst amplification. Five out of eight segments could be amplified by PCR when RT-Bst reaction was carried out using random primers (N15). Three out of eight segments could be amplified by PCR when RT-Bst reaction was carried out using eight pairs of sequence specific primers. It was also possible to selectively amplify hRSV genome sequence using multiplex primers when it was mixed with excess amount of MS2 RNA (Figure 4.13, B10). The central portion of the hRSV genome was amplified successfully using both random and multiplex primers. The lack of amplification of the terminal portions of the hRSV genome could be due to the

lower concentration of the input RNA (nanogram) or formation of strong secondary structures in the hRSV RNA. Nonspecific PCR products were amplified after PCR amplification of the RT-Bst amplified products using 8 pairs of sequence specific primers (multiplex) which could be due to the presence of the excess primers in the RT-Bst reaction.

4.5 Discussion

This study focuses on the validation of different protocols for concentrating different target sequences using 5'-biotinylated probes and enhancement of copy number of sscDNA through different types of ligation-mediated phi29-amplification. Different protocols for specific and nonspecific amplification of the bacteriophage MS2 and hRSV genomes through reverse transcription and *Bst* polymerase amplification were also validated to increase the copy number of cDNA. The novel method, RT-Bst, was found to be more efficient than the QuantiTect™ reverse transcription kit.

Sequence specific 5'-biotinylated probes and streptavidin beads were useful for concentrating lambda (λ) DNA and MS2 cDNA in this study. Detection of λ DNA was improved 100-fold (Figure 4.1) when it was concentrated using sequence specific 5'-biotinylated probes and streptavidin beads. MS2 sequence specific 5'-biotinylated probes were also found to be useful to concentrate the MS2 cDNA and remove the λ DNA from the background at picogram levels. Sequence specific probes were also found useful for concentrating Hepatitis C virus RNA by other workers. Purified RNA was used for amplification and detection of Hepatitis C virus from serum samples using fully automated PCR system, COBAS AMPLICOR, Roche Diagnostic System (Li *et al.*, 2011; Miyachi *et al.*, 2003). Isolation and concentration of episomal HPV16 DNA using sequence specific probes and magnetic beads to improve detection HPV16 by real-time PCR have been reported by Peeters *et al.* (2011). The application of magnetic beads for extraction of viral

RNA from serum sample to eliminate variety of amplification inhibitors such as heparin, haemoglobin and haeme urea has also been reported by other workers (Greenfield *et al.*, 1993; Rolfs *et al.*, 1992). In this study biotinylated probe capture assay was limited to the laboratory scale study and was not applied to any patient sample.

Ligation-mediated phi29 DNA polymerase amplification was found to be useful for nonspecific amplification of cDNA (prepared from MS2 RNA) at picogram concentrations (10^5 copy μl^{-1}) in this study. Phi29-amplification increased with the increase in the concentration of single stranded cDNA when excess amount of carrier RNA (nanogram level) was added to the reaction for T4 RNA ligase mediated phi29 DNA polymerase amplification (Section 4.3.1). In this study double stranded cDNA could be amplified from picogram concentrations (10^5 copy μl^{-1}) using T4 DNA ligase-mediated phi29 DNA polymerase amplification (Section 4.3.2). The amount of template used in this study was 10 times less than that required in the Templify™ DNA sequencing template amplification kit (Reagin *et al.*, 2003). However, in another study it was found that blunt end ligation of pUC19 (<0.1 pg reaction^{-1}) did not improve whole genome amplification (WGA) by PCR assay (Brukner *et al.*, 2006). Ligation-mediated phi29-amplification was found useful to improve detection of small genomes and fragments of nucleic acids in this study and in previous studies by other workers (Berthet *et al.*, 2008; Xu *et al.*, 2008).

CircLigase™-mediated phi29 DNA polymerase amplification of circularized single stranded cDNA (sscDNA) was also found to be useful at nanogram level of sscDNA (10^8 copy μl^{-1}) in this study. Gadkar and Filion (2011) developed a novel method for genome walking using CircLigase™-mediate phi29 DNA polymerase amplification of single stranded DNA. Successful rolling circle amplification (Section 1.8.2.3) of circular DNA using phi29 DNA polymerase (Beyer *et al*., 2005) and DNA polymerase enzyme (Liu *et al.*, 1996) has also been reported by other workers. Circularization and rolling circle amplification of ssDNA has also been

used for amplification of 5’ and 3’ cDNA ends by other workers (Polidoros *et al.*, 2006). This protocol for circularization and phi29 DNA polymerase amplification can be useful for different objectives for example, rapid amplification of cDNA ends (RACE), improve detection of target sequence and synthesis of template DNA for sequencing (Nelson *et al*., 2002).

In all assays of ligation-mediated phi29-amplification multiple bands were observed when PCR detection was used. Amplification of these multiple bands in PCR was possibly due to the effect of ligation which caused the template to form concatemers of the same sequence. However, concentration of single stranded DNA using 5’-biotinylated probes and ligation mediated phi29 DNA polymerase amplification of single stranded cDNA was not found to be more sensitive compared to the commercially available reverse transcription kit in this study. The efficiency of these methods relies on the performance of reverse transcription and preparation of cDNA using commercial kit and enzyme. Moreover, the protocols for biotinylated probe capture and ligations-mediated phi29-amplification were relatively lengthy, time consuming and laborious compared to RT-Bst amplification. Application of an automated system could be an alternative approach but can be relatively expensive and may not be affordable by diagnostic laboratories (Miyachi *et al*., 2003). It was necessary therefore to design an alternative protocol to improve detection of virus sequences.

The novel method developed in this study, RT-Bst, was found to be more effective for reverse transcription and amplification of cDNA directly from RNA template compared to reverse transcription alone (Section 4.4). Unlike reverse transcription, RT-Bst amplification not only synthesizes cDNA from MS2 RNA but also increases its copy number. Amplification of cDNA copy number after RT-Bst reaction was determined by comparing the band intensities of PCR products using RT-Bst amplicon with those of cDNA from the QauntiTect™ reverse transcription kit (QIAGEN, Crawley, UK) (Figure 4.11). RT-Bst is a novel method for isothermal amplification of nucleic acids compared to other existing methods for example,

nucleic acid sequence based amplification (NASBA) (Moore *et al*., 2010; Shan *et al.,* 2003) and signal mediated amplification of RNA technology (SMART) (Wharam *et al*., 2001). Unlike NASBA and SMART technique RT-Bst synthesizes multiple copies of cDNA from RNA template instead of making many copies of RNA. Moreover, cDNA is more stable than RNA as a template, therefore will be more suitable for handling compared to RNA templates in diagnostic laboratories. On the other hand, synthesis of multiple copies of cDNA after RT-Bst reaction will eliminate the necessity for additional reverse transcription step as is required in NASBA and SMART techniques. So, PCR detection of the pathogenic viruses after RT-Bst reaction will be cheaper than those of NASBA and SMART reactions. RT-Bst technique was found useful for amplification and detection of linear viral single stranded RNA genomes in this study. However, this technique can be applied to other forms of DNA and RNA genomes.

It was reported that NASBA and SMART techniques can work optimally at 41° C and 42° C, respectively. We found that RT-Bst amplification can be performed at 42° C and 50° C using MMuLV and RevertAid™ Premium/Maxima™ reverse transcriptase, respectively. This option of RT-Bst amplification at an elevated temperature of 50° C will help to eliminate secondary structures of RNA template to improve transcription as well as amplification of full length cDNA. RT-Bst worked efficiently and amplified different sections of hRSV genome compared to the QuantiTect™ reverse transcription kit in this study (Section 4.4.1). The performance of RT-Bst amplification for the whole genome amplification was improved when the thermostable forms of MMuLV reverse transcriptase, Maxima and RevertAid™ Premium reverse transcriptases (Fermentas, York, UK), were used. Therefore, the thermostable form of MMuLV reverse transcriptase, RevertAid™ premium reverse transcriptase (Fermentas, York, UK), was used for RT-Bst amplification of viral RNA extracted from nasal samples (Chapter 5) to validate its application for multiplex detection of pathogenic viruses of the respiratory tract. Based on the findings in this study it is proposed that RT-Bst

method can be a potentially better substitute for commercial reverse transcription kit for example, QuantiTect™ reverse transcription kit (QIAGEN, Crawley, UK).

Whole genome amplification is an important and useful method for nonspecific amplification and detection of novel virus sequences (reviewed by Ambrose and Clewley, 2006). RT-Bst PCR is a novel approach developed in this study for amplification of whole genome of RNA viruses. This approach provides the opportunity for WGA using both random and sequence specific primers (Section 4.5.3). Multiple PCR bands appeared after using cDNA as template from RT-Bst reaction using 8 pairs of sequence specific primers (Figure 4.13). These multiple bands could be due to the presence of excess primers in the RT-Bst reaction which was carried over in the subsequent PCR reaction. This technique also enables scientists to amplify viral RNA directly without going through the series of steps such as, 1st strand synthesis, 2nd strand synthesis, synthesis of blunt ended double stranded cDNA, adapter ligation, PCR amplification, cloning and sequencing (Reyes and Kim, 1991). A further modification of the WGA using the RT-Bst PCR technique from RNA viruses using random hexamers with tail sequences has been described in Chapter 6. Whole genome amplification using the RT-Bst PCR is a cheaper and simpler method for whole genome amplification compared to the SISPA technique (Section 6.5).

RT-Bst amplification was found to be the most effective protocol for synthesis and amplification of cDNA from RNA samples in a one-tube reaction from nanogram concentrations in this study. RT-Bst amplified products can be used as template for PCR detection similar to other commercially available reverse transcription kit. RT-Bst amplified product was also found to be suitable for quantitative PCR (qPCR) amplification when they were diluted 1:10 (data not shown). RT-Bst amplification can be used for producing sufficient nucleic acid material to enhance broad-spectrum pathogen detection using microarray platforms. Similar amplification of viral nucleic acids from clinical samples using phi29 DNA polymerase was found to improve detection and characterization of pathogenic

viruses in microarray analysis by other workers (Erlandsson *et al*., 2011). Although RT-Bst amplification was found useful for MS2 and hRSV in this study, a larger scale study of this technique will be necessary to validate its application to improve detection of pathogenic viruses in patient samples. In this study we determine the sensitivity of the RT-Bst technique using a qualitative PCR based assay. However, a quantitative real-time PCR assay will be more realistic to determine the sensitivity of the RT-Bst amplification. This technique was used to validate its efficiency for multiplex detection of the pathogenic viruses in 70 nasopharyngeal samples as discussed in Chapter 5.

Chapter 5

RT-Bst amplification for detection and characterization of pathogenic viruses of the respiratory tract

5.1 Introduction and aim

The developments of molecular diagnostic methods have revolutionized the detection of pathogenic viruses of the respiratory tract not only for unparalleled sensitivity of detection but also for assaying a large number of causative agents at a reasonable cost (Antonishyn and Levett, 2010; Beck and Henrickson, 2010),. This has the opportunity to better understand the clinical significance of infections involving multiple agents (reviewed by Olofsson *et al*., 2011). Conventional culture and serological techniques are routinely used for diagnosis of pathogenic viruses of the respiratory tract (Gardner and McQuillin, 1968). Although cell culture is still considered as the gold standard for virus detection it is usually too slow to be used for timely diagnosis of any infection (Leland *et al*., 2007). The use of immunofluorescence assays can speed up detection but are less sensitive (45 to 98%) compared to that of culture methods (Takimoto *et al*., 1991). Other major limitations of tissue culture methods are the requirement for complicated procedure for propagation of some viruses for example, coxsackieviruses. This method is cumbersome and diagnostic laboratories must maintain several cell lines simultaneously for propagating such viruses. Molecular biology techniques such as PCR and RT-PCR are more sensitive and rapid compared to cell culture and antigenic detection methods and hence could solve these problems.

PCR and RT-PCR are frequently used for detection and typing of respiratory tract viruses (Donofrio *et al.*, 1992; Eugene-Ruellan *et al.*, 1998; Freymuth *et al.*, 1997; Gilbert *et al.*, 1996). Cold and flu-like illness (CFLIs) is mostly caused by either single or multiple viruses associated with respiratory tract infections for example, influenza A, B and C; parainfluenza 1-4, human coronavirus (229E and OC43), human respiratory syncytial virus (hRSV), human adenovirus (hAdV) and human enteroviruses (hEV) (Turner, 1998). Human rhinoviruses are the most common cause (50-80%) of CFLIs (Arruda *et al*., 1997; Johnston *et al*., 1993). The other common pathogenic viruses responsible for an estimated 8-15% of cold and flu like illness include; human influenza viruses A, B and C; human parainfluenza

viruses 1-4; human coronaviruses 229E and OC43; respiratory syncytial viruses, human adenoviruses and human enteroviruses (Turner, 1998; Pappas *et al*., 2008; Eccles *et al*., 2007; Pizzichini *et al*., 1998). Cold infections can also be complicated by additional bacterial infection causing pharyngitis or sinusitis. Consequently multiplexed detection of a panel of common pathogenic viruses of the respiratory tract could aid in the detection, intervention and prevention of respiratory tract infections.

Multiplex RT-PCR methods are steadily being embraced by diagnostic laboratories for the detection of a complete panel of respiratory tract viruses (Aguilar *et al.*, 2000; Coiras *et al.*, 2003, 2004; Echevarria *et al.*, 1998a; Fan *et al.*, 1998; Grondahl *et al.*, 1999; Osiowy, 1998; Templeton *et al.*, 2004). There are a number of new multiplex PCR-based technologies that have been introduced by different companies such as, micro-bead suspension array (Mahony *et al*., 2007a), ResPlex technology (Brunstein *et al*., 2008), Infinity™ system (Raymond *et al*., 2009) and Jaguar™ system (Beck *et al*., 2010) for the detection of a panel of respiratory tract pathogens (Section 1.5.8). However, none of these emerging technologies have yet been approved by the FDA for clinical diagnosis and they have only been recommended for research use. These technologies also require sophisticated instruments and may not be appropriate for each and every diagnostic laboratory.

In a recent study, Raymond *et al*. (2009) developed and compared two methods – single TaqMan quantitative real-time PCR and automated microarray analysis using an Infiniti™ analyser for detection of 23 common respiratory tract viruses associated with infected and hospitalized children. The performance of microarray analysis for detection of viruses was assessed by TaqMan real-time PCR and was found to be similar detecting 94.1% of specimens as positive. The success of microarray detection depends on the hybridisation of nucleic acid probes to target nucleic acid. Therefore, often it is necessary to amplify the amount of nucleic acid in the study sample for appropriate detection in microarray analysis. Genomic nucleic acids present in a sample can be amplified by either PCR or non-PCR

based isothermal nucleic acid amplification techniques (Raymond *et al*., 2009; Wu *et al*., 2006; Arriola *et al*., 2007; Erlandsson *et al*., 2011). It is worth mentioning that isothermal whole genome amplification (phi29 amplification) technique is less error prone producing only 1 error per 10^6-10^7 bases (Esteban *et al*., 1993) and is also less biased, producing less than 3-fold bias compared to PCR-based whole genome amplification (Dean *et al*., 2002; Blanco *et al*., 1989; Hosono *et al*., 2003; Lasken *et al*., 2003). On the contrary, a PCR based whole genome amplification technique produces 1 error per 10^3 bases and introduces a bias of 10^4-10^6-fold in the representation of the amplified DNA producing a distorted representation of the starting template. Although it is possible to detect a panel of viruses using microarray technology, it is highly sophisticated and relatively expensive for individual laboratories to use for routine diagnosis of viral infections during seasonal outbreaks (Finkelman *et al*., 2007; To *et al*., 2009).

One step RT-PCR is the most commonly used technique for molecular detection of respiratory tract viruses (Bellau-Pujol *et al.*, 2005; Poddar, 2002) but is expensive compared to two-step RT-PCR as a routine diagnostic test (Sections 2.6.5). Screening a panel of viruses causing respiratory tract infections using one step RT-PCR requires more template RNA and is also more expensive (Section 5.3). The application of multiplex two-step RT-PCR requires less template RNA and is less expensive. However, the low copy number of viral genomes at femtogram levels may not be amplified by PCR leading to false negative results. Recently, a new technique, Amp-PCR (Section 1.7), has been introduced by Erlandsson *et al*., (2010) to enrich low copy of virus genomes containing linear and circular dsDNA from clinical samples. Unlike other PCR techniques, this technique combines phi29-amplification and real-time PCR amplification in the same tube. Initially, two reactions are separated by a wax layer so that they cannot mix. Phi29-amplification preceding the real-time PCR amplification increases PCR signals $100x10^6$-fold and enables detection of samples containing target nucleic acids normally below the detection limit of specific real-time PCR.

In this study, a new protocol called 'RT-Bst' was developed (discussed in Section 4.4) by combining reverse transcription and *Bst* DNA polymerase amplification to enrich for cDNAs in a one tube reaction. Amplification of cDNA from a minute amount of template RNA is expected to improve the sensitivity of subsequent detection of target sequences using singleplex and multiplex PCR (Section 4.4). It was observed in this study that RT-Bst amplification can be used for the synthesis and amplification of cDNA from the bacteriophage MS2 RNA virus genome (Section 4.4). It was demonstrated that this RT-Bst technique can be applied for the synthesis and amplification of cDNA from RNA viruses in patient samples.

Seventy nasopharyngeal samples (collected from Chelsea and Westminster Hospital, London, Section 2.1.3) were screened in this study using a multiplex OneStep™ RT-PCR kit (QIAGEN, Crawley, UK) and a newly developed protocol RT-Bst (Section 4.4) which was followed by multiplex PCR for detection of 12 common respiratory tract viruses in 3 multiplex assays (Section 2.6.6). Results obtained after multiplex RT-Bst PCR detection were compared with those of multiplex one-step RT-PCR detection to validate the performance of the newly developed RT-Bst method for detection of common respiratory tract viruses. These findings were also compared with those of the diagnostic tests performed at the Chelsea and Westminster Hospital as part of their standard operating procedures for further evaluation of the method.

5.2 Application of a RT-Bst technique for amplification of cDNA from RNA viruses in nasopharyngeal samples

RNA from the nasopharyngeal aspirates was extracted using the QIAamp™ viral RNA mini kit (Section 2.6.3). 2.5 µl of extracted RNA was amplified using the RT-Bst protocol (Section 2.6.6.1) and used for the detection of 12 common respiratory tract viruses in 3 multiplex PCR formats (Section 2.6.7). The same extracted nasopharyngeal sample RNA (2.5 µl for each multiplex reaction) was used in three

multiplex one-step RT-PCR reactions for the detection of 12 respiratory tract viruses (Section 2.6.5). This allowed a comparison to be made between the virus detection performances of each technique.

Additional primers recommended by the WHO for detection of influenza A and swine influenza A were used to screen all samples using either a singleplex one-step RT-PCR or a singleplex PCR subsequent to RT-Bst amplification. MS2 RNA was used as carrier RNA instead of the carrier RNA provided with the QIAamp™ viral RNA kit. MS2 RNA was also used as an internal control to check the performance of RNA extraction, reverse transcription and PCR detection processes.

PCR amplification bands which appeared positive for any virus were purified from the above samples using a PCR product purification column (Section 2.6.11) and the purified DNA was sequenced (Section 2.7) either using forward or reverse primers previously used for the PCR amplification. In the case where PCR was positive for both one step RT-PCR and RT-Bst amplification, either one of the PCR products was purified and sequenced. Samples positive for multiple viruses in multiplex PCR or multiplex RT-PCR were repeated in singleplex PCR or singleplex RT-PCR using corresponding virus specific primers for purification of the PCR product and sequencing. Alternatively, PCR products were cloned into a pCR 2.1 cloning vector using the TOPO-TA cloning kit (Section 2.6.8). One or two colonies obtained from the transformation of *E. coli* with plasmids (selected on LB agar containing kanamycin (30.0 µg μl^{-1}) were picked at random to confirm the presence of a cloned PCR product insert by colony PCR (Section 2.6.9). Clones positive for the desired PCR products were subsequently cultured in LB broth containing kanamycin (30.0 µg μl^{-1}) for plasmid extraction (Section 2.6.10). Purified plasmids were sequenced using M13 forward or reverse primer (Section 2.7). Sequences information was analysed using the BLASTn tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and matching with other sequences in the NCBI database for confirmation of viral sequences (Section 2.8).

The one-step RT-PCR and RT-Bst PCR techniques described in this section were used for the detection of twelve common respiratory tract viruses in three multiplex assays in seventy patient nasopharyngeal samples (Table 2.4). Prior to this, these seventy samples were also analysed by the Department of Virology, Chelsea and Westminster Hospital using immunofluorescence (IF) and/or PCR techniques. Our results were compared with that of the hospital data in Table 5.1 (samples 1-25), Table 5.2 (samples 26-50) and Table 5.3 (samples 51-70). The performances of the different techniques in terms of detecting viruses assayed are compared in these tables.

It was found that compared to one-step RT-PCR, RT-Bst PCR detected 4.3% more influenza A virus, 20% more for hRSV, 1.4% more SWA and 1.4% more hMPV. The RT-Bst PCR detection was respectively 4.3%, 1.4% and 4.2% less effective in the detection of hRV, parainfluenza 1 and influenza B. Overall RT-Bst PCR in this study was found to be 2.8% less efficient than multiplex one-step RT-PCR for detection of 12 common respiratory tract viruses.

The findings of this study were compared with those of the initial hospital diagnosis to evaluate our detection methods. Multiplex RT-Bst PCR was able to detect virus in 7.8% and 8.6% more samples compared to IF detection for hRSV and IA respectively. SWA, hRV, hMPV and parainfluenza 4 were detected in 1.4% more samples using multiplex RT-Bst PCR compared to the IF technique. Influenza B was detected only by IF technique (1.4%) and was not detected using RT-Bst PCR. Parainfluenza 1 virus was only detected using one-step RT-PCR in this study. It was not possible to detect parainfluenza 1 virus by any of the RT-Bst PCR and IF techniques. Overall detection of any virus in 70 samples tested was enhanced 17.2% using multiplex RT-Bst PCR compared to the IF method. However, it was not possible to compare RT-Bst PCR results with those of the hospital PCR results due to inadequate data available for PCR detection of the samples.

Co-infections were found in 5.6% and 2.4% of the total samples using the one-step RT-PCR and RT-Bst PCR techniques, respectively. Human respiratory syncytial

virus was found to be present in all of the co-infections detected in different samples using multiplex one-step RT-PCR and RT-Bst PCR techniques.

Table 5.1: Results for the detection of 12 respiratory tract viruses in nasopharyngeal sample 1-25 using multiplex one step RT-PCR and RT-Bst PCR technique

Sample no.	Sample Code	Chelsea and Westminster Hospital		This study	
		IF	PCR	One step RT-PCR	RT-Bst PCR
NS1	m1861375	**IA**	**H1N1**	**IA**	**IA**
NS2	W1911400	Negative	Negative	**hRSV**	**hRSV**
NS3	W188400	Negative	n/a[1]	Negative	Negative
NS4	M1881699	Positive after 5 days	n/a	Negative	**hRSV**
NS5	T2060569	Negative	Negative	Negative	Negative
NS6	1117207H	Negative	n/a	Negative	Negative
NS7	S591685	Negative	n/a	Negative	Negative
NS8	T2082489	Negative but transplant patient	n/a	Negative	**hRSV**
NS9	F1750270	Negative	n/a	**hRSV**	**hRSV**
NS10	H2002593	Negative	n/a	Negative	Negative
NS11	CID5030720	Negative	n/a	Negative	Negative
NS12	H2024910	Negative	Negative	Negative	Negative
NS13	S592481	Negative	n/a	Negative	Negative
NS14	T12077805	Negative	Negative	Negative	Negative
NS15	1200415H	Negative	**hRSV**	**hRV**	**hRV**
NS16	M1877965	Negative	Negative	Negative	Negative
NS17	T2061970	Negative	Negative	Negative	Negative
NS18	M1877977	Negative	Negative	Negative	Negative
NS19	M1861081	Negative	n/a	Negative	Negative
NS20	T2055922	Negative	n/a	Negative	Negative
NS21	T2078974	Negative	Negative	Negative	Negative
NS22	M1903348	Negative	n/a	Negative	Negative
NS23	W1890524	**hRSV**	n/a	**IA**	**IA**, **hRSV**
NS24	H2017121	Negative	n/a	Negative	Negative
NS25	1057339H	Negative	Negative	Negative	Negative

[1], not available

Table 5.2: Results for the detection of 12 respiratory tract viruses in nasopharyngeal sample 26-50 using multiplex one step RT-PCR and RT-Bst PCR technique

Sample no.	Sample code	Chelsea and Westminster Hospital		This study	
		IF	PCR	One step RTPCR	RTBst PCR
NS26	W1891748	Negative	**hRSV**	**hRV**	Negative
NS27	T2082278	Negative	**hRV**	**hRV**	Negative
NS28	T2078395	Negative	**hRSV**	**hRSV**	Negative
NS29	W1884596	Negative	Negative	Negative	Negative
NS30	W1890529	Negative	n/a[1]	Negative	Negative
NS31	M1903413	Negative	n/a	Negative	**IA***
NS32	T2082862	Negative	n/a	Negative	Negative
NS33	H2005688	Negative	Negative	Negative	**IA***
NS34	X503019	Negative	n/a	Negative	**IA***
NS35	T2082072	**hRSV**	**hRSV**	**hRSV**	**hRSV**
NS36	M1908183	Negative	Negative	Negative	Negative
NS37	W1889652	Negative	Negative	Negative	Negative
NS38	H2005727	Negative	n/a	Negative	Negative
NS39	M1856019	**hRSV**	**hRSV**	**hRSV**	**hRSV**
NS40	M1880106	Negative	n/a	Negative	Negative
NS41	T2078454	**hRSV**	n/a	**hRSV, RV**, **IA**	**IA**
NS42	M1864581	**hRSV**	**hRSV**	**hRSV, hRV**	**hRSV**
NS43	W1884898	Negative	Negative	Negative	Negative
NS44	T2080387	Negative	Negative	Negative	Negative
NS45	H2005617	Negative	Negative	Negative	Negative
NS46	T2079023	Negative	**hRSV**	**hRSV**	**hRSV**
NS47	M1902196	Negative	**IA**	**IA**	**IA**
NS48	S605599	**hRSV**	**hRSV**	**hRSV**	**hRSV**
NS49	H2023649	**hRSV**	**hRSV**	**hRSV**	**hRSV**
NS50	T2059421	Negative	**hRSV**	**hRSV**	**hRSV**

[1], not available; *Primers used according to WHO recommendation

Table 5.3: Results for the detection of 12 respiratory tract viruses in nasopharyngeal sample 51-70 using multiplex one step RT-PCR and RT-Bst PCR technique

Sample no.	Sample code	Chelsea and Westminster Hospital		This study	
		IF	PCR	One step RTPCR	RTBst PCR
NS51	X509003	Negative	Negative	Negative	Negative
NS52	W1914290	**hRSV**	n/a[1]	**hMPV, hRSV**	**hMPV, hRSV**
NS53	W1890542	Negative	Negative	Negative	Negative
NS54	T2057728	Negative	Negative	Negative	Negative
NS55	M1882085	Negative	Negative	Negative	Negative
NS56	S591988	Negative	n/a[1]	Negative	Negative
NS57	M1880948	Negative	Negative	Negative	Negative
NS58	H2015945	Negative	Negative	Negative	Negative
NS59	H2007221	Negative	n/a[1]	Negative	Negative
NS60	F1747678	Negative	n/a[1]	**Parainfluenza 4**	**Parainfluenza 4**
NS61	W1913085	Negative	**Parainfluenza 1**	**Parainfluenza 1**	Negative
NS62	F1746086	Negative	Negative	Negative	Negative
NS63	F1748369	Negative	Negative	Negative	Negative
NS64	W1890500	Negative	**Influenza B**	**Influenza B**	Negative
NS65	H2016724	Negative	**hRSV**	**hRSV**	**hRSV**
NS66	W1932015	Negative	n/a[1]	**hRSV, Influenza B**	Negative
NS67	T2078447	**hRSV**	**hRSV**	**hRSV**	**hRSV**
NS68	H2046830	**Influenza B**	**Influenza B**	**Influenza B**	Negative
NS69	M1858586	Negative	Negative	Negative	Negative
NS70	H20067…, CID4555257	Negative	Negative	Negative	Negative

[1], not available

5.3 Discussion

The application of a novel method — RT-Bst for reverse transcription and amplification of cDNA from RNA extracted from 70 nasopharyngeal samples was validated. The performance of RT-Bst PCR detection was compared with a commercially available OneStep™ RT-PCR kit (QIAGEN, Crawley, UK) to determine the feasibility of RT-Bst PCR technique as an alternative approach. Random pentadecamer primers were used in this study to increase the yield of RT-Bst amplification as it was found to be useful to improve the performance of reverse transcription in previous studies (Stangegaard *et al.*, 2006). All of the samples were detected in duplicate for both multiplex one-step RT-PCR and RT-Bst PCR in order to compare their performances for detection of 12 common respiratory tract viruses. Although the initial template RNA amount (2.5 µl) was the same for both one step RT-PCR and RT-Bst amplification, only 1.0-2.0 µl of RT-Bst amplicons were used as templates for multiplex PCR detection of 12 respiratory tract viruses. As a result, multiplex RT-Bst PCR represented 1:10 to 1:5 times less template included for PCR amplification compared to multiplex one-step RT-PCR. This is possibly one of the reasons for the observed lower efficiency of RT-Bst PCR detection in some cases.

In the study, all of the seventy samples were tested using multiplex one-step RT-PCR and RT-Bst PCR to determine the performance of RT-Bst PCR compared to the commercial OneStep™ RT-PCR kit (QIAGEN, Crawley, UK). Multiplex RT-Bst PCR was repeated an additional three times after the duplicate detection of viruses (only for the first 25 samples). It was possible to detect two more samples (4 and 8) positive for hRSV compared to the one-step RT-PCR (Table 5.1) after this repeat experiment. Detection of hRSV in sample 4 and 8 was further supported by patient information from the hospital. These two patients were organ transplant recipients and they developed hRSV infection subsequently after five days of collecting the nasopharyngeal samples. This result indicates that it was possible to detect viruses present in the samples normally below the PCR

detection limit after amplifying the cDNA copy using RT-Bst technique and by repeating the PCR for additional three times. Therefore, for all of the remaining samples (26-70), RT-Bst PCR represented 1:5 to 1:10 times less template amplification in PCR compared to one-step RT-PCR. On the other hand virus genomes could be detected successfully using one-step RT-PCR even from a sheared RNA as long as the target sequence was intact which could possibly not be amplified using RT-Bst technique as it requires relatively larger piece of RNA for RT-Bst amplification. RNA is labile and degrades easily even during the nucleic acid extraction and sample processing. However, a positive result in molecular diagnostic test may not always be associated with infection state but can be a clinically irrelevant carrier state (Brittain-Long *et al*., 2010).

It is envisaged that RT-Bst amplification becomes successful only when the template length is reasonably long (> 2.0 kb), which is a precondition for whole genome amplification of DNA using a similar enzyme, phi29 DNA polymerase (Berthet *et al.*, 2008). It was shown in a previous study that the quality of the extracted RNA was important for efficient reverse transcription which will also be necessary for RT-Bst amplification (Bustin and Mueller, 2005). Primer design, the position of the target sequence in the whole genome and the amplicon size for PCR detection were also important factors to be considered for RT-Bst PCR detection as it was also found to be critical for RT-PCR in previous studies (Mueller *et al*., 2004; Raengsakulrach *et al*., 2002; Bustin and Mueller, 2005). The central portion of the hRSV genome was found to amplify more efficiently than the terminal parts of the RNA genome as observed in previous assays (Section 4.4.1, 4.4.2, 4.4.3). This type of amplification was possibly due to the random binding of primers throughout the genome and amplifying cDNA using *Bst* DNA polymerase starting from the terminal ends towards the central part of the cDNA.

In virology research, the sensitivity of the PCR technique is determined by comparing the results with that of immunofluorescence and tissue culture (Bellau-Pujol *et al*., 2005; Hindiyeh *et al.*, 2001). In this study, it was not possible to

determine the sensitivity of the RT-Bst PCR method due to the lack of facilities in house to perform tissue culture and immunofluorescence tests. If we compare the performance of the newly designed RT-Bst PCR with the standard one-step RT-PCR technique, they were comparable. We found the performance of RT-Bst PCR better than the immunofluorescence tests performed by the hospital team on 70 samples. It may be noted that hospital samples were collected and tested 6-12 months before we detected viruses in the laboratory using multiplex RT-Bst PCR and one-step RT-PCR. This late processing of samples may cause loss of templates in the nasopharyngeal samples. A comparison of the detection of viruses by the hospital team and this study is shown in Table 5.1, 5.2 and 5.3. The sensitivity of the different assays varied for detection of different types of viruses. In a previous study Hindiyeh *et al*., (2001) used Prodesse Hexaplex multiplex PCR for the detection of pathogenic viruses of the respiratory tract. They found that detection of hRSV (91%) was less sensitive compared to the other viruses such as influenza A, influenza B and PIV1-3 showing sensitivity of 98.6%, 100% and 100%, respectively. In this study, performances of one-step RT-PCR and RT-Bst PCR were the same for detection of hRSV whereas the performance of RT-Bst PCR was 4.3% better for detection of influenza A and 4.3% less for detection of hRV.

In earlier studies, other workers used multiplex hemi-nested (Bellau-Pujol *et al*., 2005) and nested (Coiras *et al.,* 2003) PCR to confirm the sequences of the amplified products in their multiplex PCR assays. However, in this study we sequenced (Section 2.7) the amplified products after multiplex PCR assay to determine the presence of any pathogenic virus. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence was used as internal control in another study to monitor the performance of RNA extraction and PCR amplification (Bellau-Pujol *et al*., 2005). GAPDH was chosen as an internal control as it is transcribed by nasal mucous cell and would be present in all samples to monitor the assays. However, GAPDH was not found to be useful as an internal control in this study owing to the inconsistent amplification of GAPDH sequences from different samples using one-step RT-PCR and RT-Bst PCR techniques. This could be due

to the poor abundance of nasal mucosal cells or degradation of the transcribed products. Moreover, the amplicon size of GAPDH was close to that of parainfluenza 2 (in multiplex 2) (Table 2.4) in this study and it was difficult to differentiate parainfluenza 2 and GAPDH PCR bands on the same gel after multiplex PCR amplification. Consequently, it was necessary to repeat the PCR using individual primer sets to determine the presence of GAPDH or parainfluenza 2 virus. Other workers also used internal control supplied with the Promega® kit to determine the presence of any amplification inhibitors during multiplex reverse transcription nested-PCR detection of respiratory tract viruses from clinical samples (Coiras *et al.,* 2003). In this study bacteriophage MS2 RNA was used as carrier RNA instead of the total RNA provided with the QIAamp RNA mini kit (QIAGEN, Crawley, UK). MS2 RNA was also used as internal control to check the performance of the test. Unlike GAPDH sequence as internal control, MS2 sequence consistently produced positive bands and reflected upon the performance of tests starting from extraction to amplification. MS2 sequence was detected from each and every sample by singleplex PCR in addition to three multiplex one-step RT-PCR and RT-Bst PCR detection. If it was not possible to amplify the MS2 sequence properly in any of the samples, possibly due to the presence of any contaminants or inhibitors, the whole process was repeated to obtain more acceptable results. MS2 RNA was also used as a control RNA by Mahony *et al*. (2007a) during detection of a panel of twenty human respiratory tract viruses using multiplex PCR and a fluid microbead-based assay. However, in a previous study it was found that only 6% of researchers test their nucleic acid samples for the presence of inhibitors (Bustin, 2005).

Appropriate primer design to amplify a suitable size of product could be useful to improve RT-Bst PCR detection. It was not possible to design and optimise new primer pairs in this study for pathogenic viruses of the respiratory tract due to the lack of facility to propagate viruses. In the case of influenza A virus detection two different primers were used and one of them was a degenerate primer recommended by WHO for detection of influenza A virus (Appendix Table A1). The

RT-Bst PCR amplification and detection was enhanced when both the primers were used for detection of influenza A virus in samples 31, 33 and 34 (Table 5.2). Ideally, it is necessary to use positive control stocks of viruses to evaluate the performance of any method (Bellau-Pujol *et al*., 2005). However, due to the restrictions on propagating pathogenic viruses in this study consequently tissue culture media positive for hRSV, parainfluenza 2 and influenza B, (provided by Saint Marys Hospital, London), were used to check the performance of multiplex one-step RT-PCR and RT-Bst PCR amplification. Primers used for the detection of hRV in this study were shown to amplify over 60 serotypes (Savolainen *et al.*, 2002) which under represents detection of the full range of 100 genotypes of hRV.

Nuclease contamination of viral RNAs is very common when they are present in any live sample e.g. intraocular fluids (Wiedbrauk *et al*., 1995) and urine (Chernesky *et al*., 1997). This might lead to false-negative results especially during detection by PCR. The quality of template RNA is also important for reverse transcription (Berthet *et al.*, 2008) as well as important for RT-Bst amplification. In order to carry out multiple tests from the same sample, viral samples and extracted RNA were aliquoted upon receipt to avoid multiple freeze-thaw cycle. In this study it was found that the performance of nucleic acid extraction and PCR detection varied with the variation in the sample for example, presence of host cells and virus types. It was also observed that RT-Bst amplification was more efficient when RNA was used immediately after extraction of hRSV RNA from tissue culture media and commercially available MS2 RNA (Section 4.4). Adding MS2 RNA as carrier RNA instead of the carrier RNA provided by Qiagen performed better for RT-Bst amplification and PCR detection of viruses which could be due to the purity and linearity of MS2 RNA over the total RNA provided with the kit (data not shown). One drawback with the RT-Bst amplification was that the reaction was found to be more sensitive when it was set up using newly purchased enzymes and the reaction efficiency gradually dropped with the time and use of the enzymes (data not shown).

A super high speed quantitative real time PCR has been introduced recently by Sakurai *et al.* (2011) for rapid typing of influenza virus in less than 20 minutes. However, it normally requires approximately 2 hours for a qPCR run using other thermal cyclers. This high speed qRT-PCR takes the advantage of newly designed heating system in the block. Although the authors used RNA transcripts from reconstructed plasmids and tissue culture fluid for validating the methods, their approach was very sensitive and could detect 1 copy of RNA transcript reaction^{-1} and 10^{-1} pfu reaction^{-1} in tissue culture fluid. This rapid method would be very useful for monitoring the outbreak and control the spread of respiratory tract infections especially in the hospital environment. A pre-enrichment of cDNA using the RT-Bst technique could be useful for such rapid detection of any pathogenic viruses using qRT-PCR from broad range of clinical and environmental samples.

Screening patient samples for a panel of viruses could be helpful for appropriate and timely management of spread of infections and unwanted prescription of antibiotics (Lee *et al*., 2011; Dosh, 2000). Although RT-Bst amplification takes a little longer than one step RT-PCR, cost wise it was significantly cheaper in consumables than multiplex one-step RT-PCR reaction. Considering the cost effectiveness of RT-Bst amplification and PCR detection additional pathogenic viruses such as enterovirus and bocavirus could be screened using this technique. Screening a panel of respiratory tract viruses will help control spread and intervention of respiratory tract infections (reviewed by Olofsson *et al*., 2011).

Taken together, the evidence and findings in this chapter suggests that RT-Bst amplification could be a more sensitive and cheaper option for rapid detection of a panel of respiratory tract viruses commonly causing human respiratory tract infections. RT-Bst amplification is a competitive method for reverse transcription and amplification of cDNA directly from an RNA sample. A more detailed study is needed for further optimization and validation of the method using different types of virus genomes at various temperatures before applying it on clinical samples.

However, RT-Bst amplification technique has potential to be used as an alternative to any commercially available reverse transcription kit.

Chapter 6

Amplification of genomic RNA of viruses using a novel method—RT-Bst SPA

6.1 Introduction and aims

The detection of currently unknown pathogenic viruses from clinical samples is one of the most challenging problems in diagnostic molecular virology. Known viruses can be detected using an appropriate primer pair in PCR amplification. However, primers specific for known viruses may not bind specifically to nucleic acids of currently unknown and new variants of viruses leading to false negative results (Zhang *et al.*, 2011; Quan *et al*., 2007). Therefore a sequence independent approach will be required for detection of these new types of viruses. Whole genome amplification (Section 1.6) is a sequence independent approach for discovery, detection, and characterization of novel pathogenic microorganisms from a limited amount of DNA (Hutchison and Venter, 2006; Lasken, 2007; Hughes *et al*., 2005). The entire genome of pathogenic microorganisms can be amplified broadly in two ways, using PCR based techniques and non-PCR based isothermal amplification techniques (Section 1.6). PCR based whole genome amplification uses random or degenerate primers for PCR amplification of the whole genomes. In another approach genomic DNA is digested to ligate with the adapters for subsequent amplification of the whole genome from the adapter sequences. Isothermal amplification of nucleic acids can use phi29 or *Bst* DNA polymerase, random primers and nucleotides to amplify the nucleic acids using multiple displacement activity. Random primers bind to different areas of the denatured template and the polymerases extend the primers and synthesize the complementary strand. Random primers proceed and displace the 5'-end of the upstream strands. This reaction is repeated for the displaced strands producing a hyper-branched amplification of DNA (Figure 1.4). NASBA is another method used for the amplification of an RNA template (Guatelli *et al*., 1990) (Figure 1.1).

Many new virus types have been identified with the development of molecular techniques in the last two decades (Section 1.1). Sequence independent single primer amplification (SISPA) is a PCR based method for the amplification of whole genomes and library preparation for sequence based detection of any

uncharacterized viruses (Section 1.8.1.2). SISPA requires the synthesis of double stranded cDNA (from ssRNA genomes) and ligation of adapters at both ends of digested double stranded cDNA. Fragments of dsDNA flanked with primer sequences are amplified using a single primer (Figure 1.3). Allander *et al.* (2007) discovered new types of human polyomavirus using sequence independent single primer amplification (SISPA). The SISPA technique, in combination with immunoscreening was also used for characterization of Norwalk virus from faeces samples (Matsui *et al*., 1991) and human astrovirus from tissue culture supernatants (Matsui *et al*., 1993). DNase-SISPA is a modified version of SISPA technique that uses a DNase step to remove contaminating human and other free DNA molecules. Virus nucleic acids that remain protected within their capsids are subsequently extracted and amplified using the SISPA technique. Allander *et al*. (2001) identified new parvoviruses from serum sample using the DNase-SISPA technique.

Phi29-amplification of the whole genome using multiple displacement amplification (MDA) (Section 1.8.2.1) can produce 100 µg of DNA from a few microliters of blood (Hosono *et al*., 2003) or even from fingerprints (Sorensen *et al*., 2004). It is possible to replicate 99.8% of the template genome with low amplification bias (less than three-fold) in the representation of different regions of the genome using MDA (Dean *et al*., 2002; Hosono *et al*., 2003). Amplicons generated in MDA are high-molecular-weight DNA (Dean *et al*., 2002 and 2001) of 2 to 100 kb in length. On the contrary, PCR-based whole genome amplification methods are highly biased (amplification bias 10^4-10^6-fold) and produces shorter products (only a few hundred base pairs in length) (Dean *et al*., 2002). Multiple displacement amplification (MDA) products have been used for restriction fragment length polymorphism (RFLP) detection (Luthra *et al*., 2004, Dean *et al*., 2002), gene rearrangement and DNA sequencing (Mai *et al*., 2004; Paez *et al*., 2004; Lovmar *et al*., 2003; Detter *et al*., 2002; Dean *et al*., 2001). Phi29 DNA polymerase is a high fidelity (Nelson *et al*., 2002; Esteban *et al*., 1993) enzyme and has been found to be useful for genotyping of SNPs and point mutations (Baker *et al*., 2004; Paez

et al., 2004; Hosono *et al*., 2003; Lovmar *et al*., 2003; Tranah *et al*., 2003; Dean *et al*., 2002).

The large fragment of *Bst* DNA polymerase is another enzyme that amplifies the whole genome using multiple displacement amplification technique with less bias (less than three-fold) (Lage *et al*., 2003; Dean *et al*., 2002; Rook *et al*., 2004). *Bst* DNA polymerase amplifies the whole genome in a similar mechanism to phi29 DNA polymerase. Unlike phi29 DNA polymerase *Bst* DNA polymerase does not have 3' to 5' exonuclease activity (Aliotta *et al*., 1996). *Bst* DNA polymerase was also used for the amplification of DNA from formalin-fixed paraffin-embedded tissues (Aviel-Ronen *et al.*, 2006). *Bst* DNA polymerase amplification can be used for isothermal nucleic acid amplification of the human genome for comparative genomic hybridization array (Aviel-Ronen *et al*., 2006).

Whole genome sequencing and microarray analysis require higher concentrations of DNA which can be amplified from limiting amounts of clinical samples using any of the PCR or non-PCR based whole genome amplification methods (Erlandsson *et al*., 2011; Mejlhede *et al*., 2009, Pan *et al*., 2008; Palacios *et al*., 2007). The whole genome amplified DNA can be either cloned to make a library for analysing the sequences using the Sanger sequencing method or sequenced directly using Next Generation Sequencing (NGS) techniques for example, 454 (Roche), Solexa (Illumina) and SOLiD (ABI) (Section 1.8.4). Although the NGS techniques produce millions of sequences in a single round of operation (Margulies *et al*., 2005), they can vary in their read lengths based on the type of technology used. Both the Sanger and NGS approaches are relatively expensive to determine the whole genome sequences and to detect new types of sequences. Microarray analysis can be a cheaper approach for detection of known and undescribed viruses from clinical samples (Wang *et al*., 2003; Gardner *et al*., 2010) (Section 1.5.7). In a recent study, Nicholson *et al*., (2011) successfully demonstrated the utility of a panviral microarray (Virochip platform) for detection of swine viruses in both cell culture supernatant and clinical samples. This Virochip platform contains highly

conserved 70 mer sequences from every sequenced viral genome for simultaneous detection of many known, novel and emerging viruses (Wang *et al*., 2003). Gardner *et al*. (2010) designed a pan microbial detection array and a novel statistical analysis method for detection of all known viruses (including bacteriophages), bacteria and plasmids. This array is more robust compared to the previous microarray analysis because it uses more family specific probes per target, 50 for viruses and 15 for bacteria in spite of 2-10 probes per target in previous assay. This array is more up to date than the previous array (Wang *et al*., 2003) because the probe sequences are designed using a more recent sequence database. Both of these arrays have the potential to detect and characterize known and novel sequences of pathogenic microorganisms.

Subtractive hybridization has also been found to be useful for detection of new viruses such as torque teno virus (Muerhoff *et al.*, 1997; Nishizawa *et al*., 1997) but it is relatively lengthy and may take weeks to analyse each sample. Another approach for WGA is particle associated nucleic acid PCR (Stang *et al*., 2005) that requires separation of virus particles from large volumes (11 ml) of sample through sucrose gradient centrifugation and synthesis of cDNA from viral RNA. In the particle associated nucleic acid PCR method virus sequences are amplified using tailed random primers to generate different sizes of amplicons. These PCR products are subsequently cloned for the preparation of a whole genome library. Although SISPA (Lambden *et al.*, 1992 and 1995) and virus-discovery-cDNA-AFLP (VIDISCA) (Van der Hoek *et al.*, 2004) are widely used for sequence independent amplification and detection of new variants of existing viruses, they are laborious and time consuming. In these two techniques RNA is reverse transcribed to single stranded cDNA and the second strand is synthesized. Blunt ends of double stranded cDNA were generated through the use of T4 DNA polymerase. Prepared double stranded cDNA is either ligated directly to blunt end adaptors or digested with suitable enzymes to ligate them to suitable adapters. Subsequently, reactions are purified to be used for PCR amplification using primer sequences from the

adapters (Reyes and Kim, 1991). However, an alternative, brief protocol could be more useful to save time and money.

In this chapter a novel protocol (RT-Bst SPA) has been developed to minimize the steps, time and cost for whole genome amplification (Figure 6.1; Table 6.5). In this approach, cDNA was prepared from RNA using tailed random primers, 5'-GACCATCTAGCGACCTCCACMNNMNM-3' (Stang *et al.*, 2005) instead of random primers during RT-Bst amplification. Complementary DNA is prepared and amplified simultaneously from RNA. In this process 3' of tailed random primers bind initially to RNA and subsequently to the synthesized cDNA and continue synthesis of the complementary strands. Consequently all of the hyper-branched cDNA contains the tail sequences at the 5'-end after RT-Bst amplification. RT-Bst amplified products can be used as a template for PCR based whole genome amplification using the tail sequence of random primers (5'-GACCATCTAGCGA CCTCCAC-3') as a single primer (RT-Bst SPA) (Stang *et al.*, 2005). The RT-Bst SPA process is explained in the following sections and figure 6.1. Amplified PCR products can either be cloned in suitable vectors for making a library or used for Next Generation Sequencing (Section 1.8.4). Purified MS2 RNA was used in this study as a control RNA to validate this protocol. Consequently, this protocol was applied on tissue culture extracts positive for hRSV, parainfluenza 2 and influenza B. Two patient samples positive for swine influenza A (sample 23) and hRSV (sample 39) were also amplified using this technique to evaluate this approach.

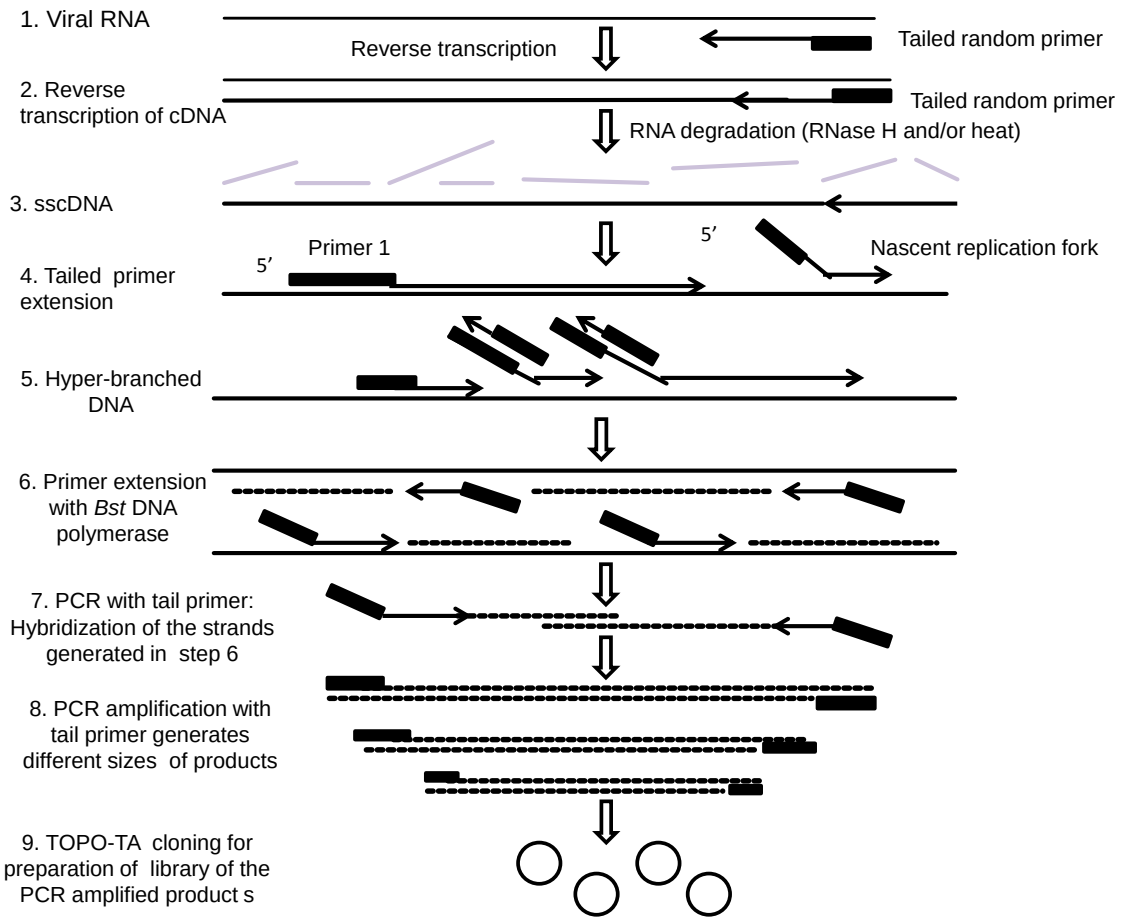

Figure 6.1: Basic steps in RT-Bst single primer amplification (RT-Bst-SPA) of viral RNA. 1: Viral RNA, 2: Reverse transcription and synthesis of cDNA using tailed primer, 3: Degradation of RNA from cDNA:RNA hybrid through RNase H activity or heat, 4: Binding and extension of the tailed primers, 5: Synthesis of hyper-branched DNA with *Bst* DNA polymerase activity, 6: Primer extension with *Bst* DNA polymerase, 7: Hybridization of the DNA strands with tail primer from step 6, 8: PCR amplification of different sizes of DNA using tail sequence as a primer. 9: TOPO-TA® cloning of the amplified PCR products to make a whole genome library.

6.2 Whole genome amplification using RT-Bst single primer amplification (RT-Bst SPA)

6.2.1 Sample processing and DNase treatment

DNase treatment (Allander *et al.*, 2001 and 2007) is necessary to remove any contaminating host DNA before whole genome amplification from clinical samples can be performed. It is expected that different samples will contain different amounts of host cells, cell free DNA and other cellular materials including microbial normal flora. An optimum DNase treatment of any sample containing viruses will vary depending on the presence of host and other DNA molecules in different samples.

Commercially available MS2 RNA was used (as control) to evaluate the protocol for whole genome amplification using RT-Bst SPA. As the MS2 RNA was already purified a DNase treatment was not required for RT-Bst SPA amplification from MS2 RNA. Moreover free RNA is more labile than viral RNA protected by a capsid and degrades easily. Tissue culture extracts positive for influenza B, parainfluenza 2 and hRSV were used for whole genome amplification using this protocol. Patient samples 23 and 39 previously determined to be positive for swine influenza A virus and hRSV, respectively, by multiplex one-step RT-PCR and RT-Bst PCR (Table 5.2a and 5.2b) were used for whole genome amplification. Initially these samples were centrifuged at 14000 *g* in a bench top centrifuge for 5 minutes to remove cellular debris. Then samples were treated with DNase (Baseline Zero™, Epicentre Biotechnologies, Cambridge, UK) according to the protocol optimized in this study as shown in table 6.1 for amplification of RNA using RT-Bst SPA.

Table 6.1: Digestion of extracellular DNA using Baseline Zero™

Reaction component	Reaction volume
Tissue culture fluid	50.0 µl
10X baseline zero reaction buffer	6.0 µl
Baseline Zero™ (1.0 U μl^{-1}) (Epicentre Biotechnology, Cambridge, UK)	2.0 µl
Nuclease free water	2.0 µl
Total volume	60.0 µl
Incubation	37º C for 10 minutes

6.2.2 RNA extraction from tissue culture media and nasopharyngeal samples

Viral RNA was extracted immediately after DNase (Baseline Zero™) treatment using either QIAamp viral RNA or QIAamp Blood DNA mini kit (QIAGEN, Crawley, UK) (Section 2.6.3) following the manufacturer's guideline with the exclusion of carrier RNA in the case of the QIAamp viral RNA mini kit. No carrier RNA was added during this extraction process in order to avoid amplification of unwanted sequences and to improve amplification of desired viral RNA after WGA.

6.2.3 RT-Bst amplification

Commercially available MS2 RNA and RNA extracted from tissue culture media positive for hRSV and influenza B were immediately amplified by RT-Bst protocol using tailed random primers, 5'-GACCATCTAGCGACCTCCACMNNMNM-3', here N represents A, T, G or C and M represents A or C (Section 2.6.6) (Stang *et al.*, 2005), instead of the pentadecamer random primers (Appendix Table A1) (Stangegaard *et al.*, 2006) as described in section 2.6.6. This tailed random primer

was used instead of the N15 primer so that double stranded cDNA prepared after RT-Bst amplification could be primed with the tail sequence added to the 5'-end of all of the amplified sequences. These tail sequences would aid in single primer amplification of the RT-Bst amplified products as discussed in section (6.2.4).

6.2.4 Single primer amplification of RT-Bst amplified products

RT-Bst amplified product generated using the tailed random primers (Section 6.2.3) was immediately used as a template for PCR reaction as described in section 2.6.7.2. The tail sequence of the random primers (5'-GACCATCTAGCGACCTC CAC-3', Stang *et al*., 2005) was used as a primer during PCR amplification (Figure 6.1). The same primer was used as forward and reverse primers for PCR amplification. The total volume of the PCR reaction was scaled up to 60 µl adding 6.0 µl of the RT-Bst amplified product as template. The PCR conditions were the same as described in section 2.6.7.2.

6.2.5 TOPO-TA cloning and preparation of genomic libraries

RT-Bst SPA product produced after whole genome amplification (Section 6.2.4) was resolved on an agarose gel (Section 2.6.1) for visualisation of the amplified products (Figure 6.2). RT-Bst SPA products were then purified through a DNA purification column (Section 2.6.11) and cloned into TOPO-TA cloning vector (Section 2.6.8) to make libraries for MS2, parainfluenza 2, influenza B, hRSV and patient samples 23 and 39 (Figure 6.2).

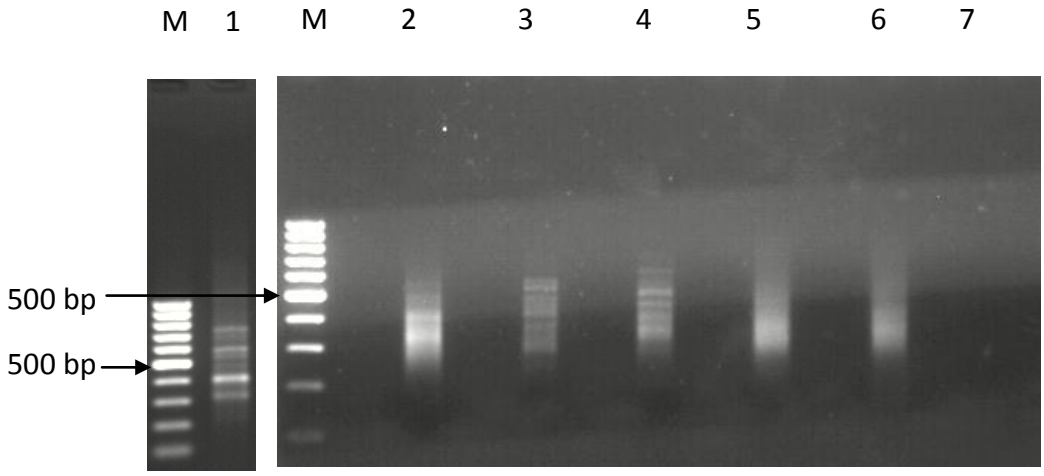

Figure 6.2: Whole genome amplification of MS2 RNA and virus RNA extracted from known positive samples using RT-Bst SPA. M: 100 bp DNA ladder (Fermentas, York, UK); Lane 1: bacteriophage MS2 RNA (20 ng); Lane 2: patient sample 23 positive for swine influenza A; Lane 3: tissue culture fluid positive for Influenza B; Lane 4: tissue culture fluid positive for parainfluenza 2; Lane 5: tissue culture fluid positive for hRSV; Lane 6: patient sample 39 positive for hRSV; Lane 7: negative control.

Subsequently, 10 colonies were picked randomly from the kanamycin (30 µg ml^{-1}) containing Luria Bertani agar plates and confirmed for the presence of inserted sequence in the vector by colony PCR (Section 2.6.9; Figure, 6.3). Clones positive for the inserted sequence by PCR were further propagated in Luria Bertani broth containing kanamycin (30 µg ml^{-1}) for the extraction of plasmid (Section 2.6.10).

After colony PCR, amplified products were separated on an agarose gel (Figure 6.3). Different sizes of cloned inserts were present in the whole genome libraries generated for different viruses. Smaller bands appeared to be preferably cloned over larger bands. This was determined when the sizes of the whole genome amplified products (Figure 6.2) were compared with those of the cloned inserts

(Figure 6.3). However, in some cases PCR products that were amplified to a greater extent were cloned more easily over less amplified products (Figure 6.2, and 6.3 for MS2). It could be possible to excise different sizes of discrete bands after appropriate DNase treatment and clone them to generate a library containing different sizes and diversity of DNA sequences instead of column purifying all the different sizes of RT-Bst SPA products.

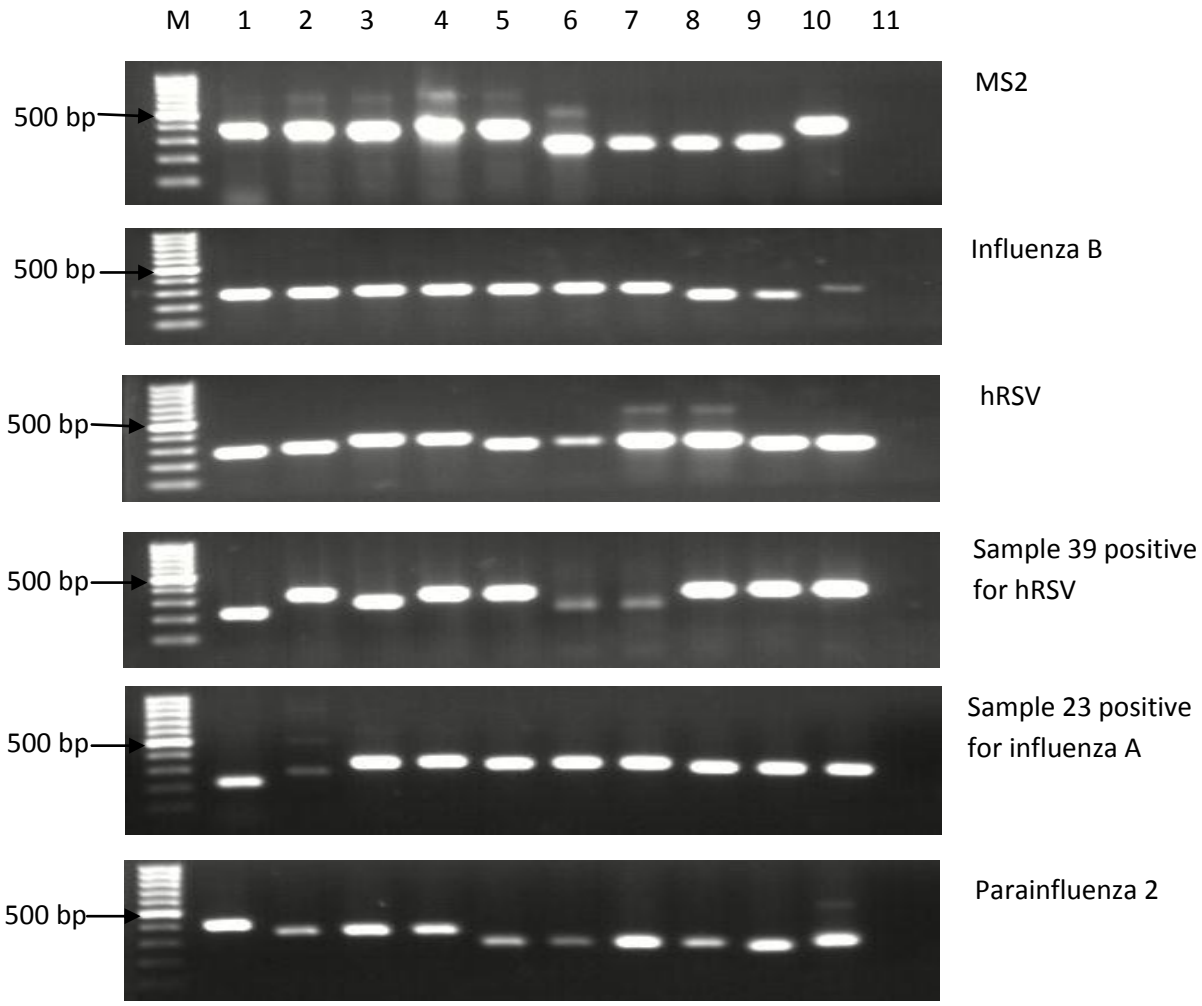

Figure 6.3: Colony PCR detection of inserts in 10 clones randomly picked from different libraries generated for different virus genomes. M: 100 bp DNA ladder (Fermentas, York, UK); Lanes 1 to 10: randomly picked colonies 1 to 10; Lane 11: negative control.

6.3 Analysis of sequences from the libraries prepared from RNA virus genomes

Three out of six libraries prepared from the RNA viruses were sequenced (Section 2.7) to check the initial performance of RT-Bst SPA for whole genome

amplification (WGA). Sequences from the clones were compared using the BLASTn tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to match them with those available in the public database (Section 2.8 and Table 6.2, 6.3 and 6.4).

Sequences of influenza B clones 1 and 2 showed partial similarities with segment 1 of influenza B when they were aligned with influenza B genomic sequences (Table 6.2). However, the sequences of influenza B clones 1 and 2 showed similarity with eukaryotic 28S rRNA sequences when aligned with all other sequences (Table 6.2). Similarly, sequences from Influenza B clones 3, 4 and 5 showed partial similarities with segment 8 of influenza B when the sequences were aligned with only influenza B genomic sequences; otherwise they did not match with any other sequence in the database (Table 6.2). Sequences of influenza B clones 6 to 9 showed partial similarity with eukaryotic sequences and clone 10 did not match with any other sequence in the database when they were aligned with other sequences (Table 6.2).

Table 6.2: BLASTn analysis of the cloned sequences from the influenza B library for sequence homology in the database

Clones	BLASTn analysis of the cloned sequences of Influenza B	
	Matched with all other sequences **Query coverage (QC, %), E-value (E), Maximum identity (MI, %)**	**Matched with influenza B sequences** **Query coverage (QC, %), E-value (E), Maximum identity (MI, %)**
Clone 1	Uncultured-Eukaryotic-28S-rRNA (232 bp) QC= 94, E= 9e-91, MI=90	Influenza B segment 1 (23 bp) QC= 8, E=0.19, MI=91
Clone 2	Uncultured-Eukaryotic-28S-rRNA (234 bp) QC= 93, E= 1e-95, MI=91	Influenza B segment 1 (23 bp) QC=8, E=0.19, MI=91
Clone 3	No match (236 bp)	Influenza B segment 8 (27 bp) QC=9, E=2.3, MI=89
Clone 4	No match (236 bp)	Influenza B segment 8 (27 bp) QC=9, E=2.3, MI=89
Clone 5	No match (236 bp)	Influenza B segment 8 (27 bp) QC=9, E=2.3, MI=89
Clone 6	Partially eukaryotic otherwise no match (243 bp)	No match
Clone 7	Partially eukaryotic otherwise no match (243 bp)	No match
Clone 8	Partially eukaryotic otherwise no match (189 bp)	No match
Clone 9	Partially eukaryotic otherwise no match (189 bp)	No match
Clone 10	No match (243 bp)	No match

The sequence of hRSV clone 1 showed similarity with human chromosome 15 sequence when it was aligned with other sequences (Table 6.3). However, clone 1 showed partial similarity with hRSV sequence when aligned with the hRSV genomic sequence only. Sequences of clones 2 to 5 showed similarity with human

chromosome 17 sequence. Sequences of clones 6 to 8 matched with human chromosome 14 when aligned with the other sequences and partially matched with hRSV when aligned with hRSV genomic sequence only. Sequences of clones 9 and 10 matched with human chromosome 8 sequence (Table 6.3).

Table 6.3: BLASTn analysis of the cloned sequences from hRSV library for sequence homology in the database

Clones	BLASTn analysis of the cloned sequences of hRSV	
	Aligned against all available sequences **Query coverage (QC, %), E-value (E), Maximum identity (MI, %)**	**Aligned against hRSV sequences**
Clone 1	Human chromosome 15 (230 bp) QC=84, E=2e-112, MI=100	Partial hRSV (18 bp) QC=6, E=0.052, MI=100
Clone 2	Human chromosome 17 (246 bp) QC=85, E=4e-121, MI=100	No match
Clone 3	Human chromosome 17 (287 bp) QC=93, E=3e-143, MI=97	Not match
Clone 4	Human chromosome 17 (287 bp) QC=93, E=3e-143, MI=97	Not match
Clone 5	Human chromosome 17 (246 bp) QC=85, E=4e-121, MI=100	Not match
Clone 6	Human chromosome 14 (262 bp) QC= 87, E= 6e-132, MI=100	Partial hRSV (18 bp) QC=7, E=0.21, MI=91
Clone 7	Human chromosome 14 (262 bp) QC= 87, E= 6e-132, MI= 100	Partly hRSV (18 bp) QC=7, E=0.21, MI=91
Clone 8	Human chromosome 14 (262 bp) QC= 87, E= 6e-132, MI= 100	Partly hRSV (18 bp) QC=7, E=0.21, MI=91
Clone 9	Human chromosome 8 (233 bp) QC= 91, E= 2e-111, MI= 97	Did not match
Clone 10	Human chromosome 8 (233 bp) QC= 91, E= 2e-111, MI=97	Did not match

The sequencing results from the tissue culture samples positive for hRSV and influenza B showed that some of the cloned sequences showed similarities to either human genomic sequences or eukaryotic sequences. There was only partial similarity to hRSV and influenza B sequences (Table 6.2 and 6.3). The presence of such unrelated sequences in the library was possibly due to poor DNase treatment (2 U). Some of the sequences did not match with any sequences in the database which possibly indicates non-specific amplification during PCR. The rest of the samples such as tissue culture samples positive for parainfluenza 2 and patient samples 23 and 39, were not analysed through sequencing, although libraries were generated from them. After analysis of the sequences from the clones prepared from MS2 RNA, it was found that all of the clones (ten) were positive for MS2 bacteriophage sequence (Table 6.4). This result indicates that if the desired RNA concentration can be increased significantly over the other types of nucleic acid sequences in the sample through appropriate DNase treatment, RT-Bst SPA can be more useful for cloning and sequencing desired sequences.

It was found that WGA using RT-Bst SPA was effective for amplification of MS2 genomic RNA and all of the clones were positive for MS2 genomic sequences. Different parts of the MS2 genome was amplified in this technique and all of these were within 217-329 bp in sizes (Table 6.4). Four out of ten library sequences were partially positive for influenza B and hRSV genomic sequences for libraries prepared from influenza B and hRSV, respectively. The lengths of the cloned sequences for influenza B and hRSV libraries were found to fall between 190-244 bp and 231-288 bp, respectively.

Table 6.4: BLASTn analysis of the cloned sequences from MS2 library for sequence homology in the database

Clones	BLASTn analysis of the cloned sequences of MS2	
	Matched with all other sequences **Query coverage (QC, %), E-value (E), Maximum identity (MI, %)**	**Matched with MS2 sequences** **Query coverage (QC, %), E-value (E), Maximum identity (MI, %)**
Clone 1	MS2 genome 2592-2911 (320 bp) QC=89, E=6e-167, MI= 99	MS2 genome 2592-2911 (320 bp) QC=89, E=6e-167, MI= 99
Clone 2	MS2 genome 2592-2911 (320 bp) QC=89, E=2e-163, MI= 99	MS2 genome 2592-2911(320 bp) QC=89, E=5e-168, MI= 99
Clone 3	MS2 genome 2590-2911 (322 bp) QC=89, E=6e-165, MI= 100	MS2 genome 2590-2911 (322 bp) QC=89, E=1e-169, MI= 100
Clone 4	MS2 genome 2590-2918 (329 bp) QC=92, E=3e-168, MI= 99	MS2 genome 2590-2918 (329 bp) QC=92, E=7E-173, MI=99
Clone 5	MS2 genome 2590-2918 (329 bp) QC=92, E=7e-173, MI=99	MS2 genome 2590-2918 (329 bp) QC=92, E=3e-168, MI=99
Clone 6	MS2 genome 1154-1370 (217 bp) QC=83, E=4e-104, MI=98	MS2 genome 1154-1370 (217 bp) QC=83, E=2e-99, MI=98
Clone 7	MS2 genome 1154-1370 (217 bp) QC=83, E=3e-103, MI=99	MS2 genome 1154-1370 (217 bp) QC=83, E=6e-108, MI=99
Clone 8	MS2 genome 1154-1370 (217 bp) QC=83, E=2e-99, MI=98	MS2 genome 1154-1370 (217 bp) QC=83, E=4e-104, MI=98
Clone 9	MS2 genome 1154-1370 (217 bp) QC=83, E=6e-108, MI=99	MS2 genome 1154-1370 (217 bp) QC=83, E=3e-103, MI=99
Clone 10	MS2 genome 2590-2918 (329 bp) QC=92, E=3e-168, MI=99	MS2 genome 2590-2918 (329 bp) QC=92, E=7e-173, MI=99

6.4 DNase treatment to improve the performance of whole genome amplification (WGA)

The Baseline Zero™ (Epicentre Biotechnology, Cambridge, UK), a DNase, was used in this study for digestion of cell-free DNA before extraction of viral RNA and amplification of the whole genome using RT-Bst SPA. Baseline Zero™ DNase was used in this study for its improved efficiency over the commonly used pancreatic DNase I for digestion of DNA. According to the manufacturer's information Baseline Zero™ degrades both the ssDNA and dsDNA to mononucleotides whereas the DNase I degrades both the ssDNA and dsDNA to short oligonucleotides and mononucleotides. The cell-free DNA was digested using Baseline Zero™ after incubating at 37 °C for 10 minutes following the manufacturer's instructions in this study. 10 µl of nasopharyngeal sample was treated individually with 10, 20, 30, and 40 U of Baseline Zero™ (Epicentre Biotechnology, Cambridge, UK) following the protocol provided by the manufacturers (Table 6.1). Subsequently, RNA was extracted from nasopharyngeal sample 65 (Section 6.2.2) and amplified by RT-Bst SPA (Sections 6.2.3 and 6.2.4; Figure 6.1). It was found that more discrete bands appeared at 30 U and 40 U of Baseline Zero™ treatments, which indicate that an optimum DNase treatment can improve the amplification of the desired sequences (Figure 6.4). A similar result of amplification of discrete bands after optimum DNase I treatment was reported by other workers (Allander *et al*., 2001). However cloning of these discrete bands could be useful to improve the performance of RT-Bst SPA (Figure 6.4, L3 and L4). Appropriate optimization of the DNase treatment can be done in future studies before amplification of the genomic RNA using RT-Bst SPA method.

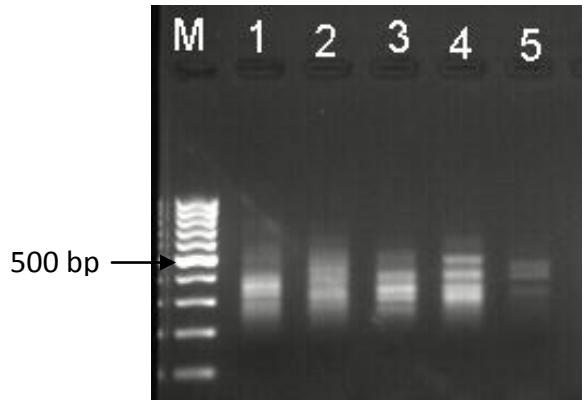

Figure 6.4 Effect of DNase (Baseline Zero™) treatments before WGA using RT-Bst SPA from sample 65. M: 100 bp DNA ladder (Fermentas, York, UK); Lane 1: Baseline zero 10 U; Lane 2: Baseline zero 20 U; Lane 3: Baseline zero 30 U; Lane 4: Baseline zero 40 U; Lane 5: negative control.

6.5 Discussion

This study was designed to develop and evaluate a method for whole genome amplification (WGA) and the resulting technique was named RT-Bst SPA. Using this method, WGA was found to be effective in the amplification of purified MS2 RNA, but its performance varied when it was applied to tissue culture samples positive for influenza B and hRSV.

Although different sizes of bands between 200 and 800 bp appeared after WGA using RT-Bst SPA, only the smaller sized PCR products (between 200 and 300 bp) were preferentially cloned (Section 2.6.8). Consequently, this did not represent the range of different sizes of cDNA amplified in RT-Bst SPA. A smear of different sizes of PCR products were observed on the gel (Figure 6.2). This could be due to inadequate DNase treatment and presence of host DNA in samples before RT-Bst SPA amplification. To overcome this problem a DNase treatment was performed using 10, 20, 30 and 40 U of Baseline Zero™ (Epicentre Biotechnology,

Cambridge, UK) to determine its effect on WGA. Nasopharyngeal sample 65, which was positive for hRSV by one-step RT-PCR was used in this experiment (Table 5.2c). It was found that more discrete bands appeared after treating sample 65 using 30 U and 40 U of DNase before RT-Bst SPA amplification. It was understood that an optimum DNase treatment could eliminate undesired sequences for example, host and other DNA present in the sample. Appropriate DNase treatment could be helpful to generate a more representative library from the whole genome of viruses.

It was not possible to optimize the DNase treatment on more samples due to limited time available for this study. However, RT-Bst SPA of whole genome can be used for either high throughput sequencing or microarray analysis. High throughput sequencing can be performed from the RT-Bst SPA amplified products using the tail sequence of random primers (5'-GACCATCTAGCGACCTCCAC-3', Stang *et al*., 2005). In this study a Sanger sequencing approach was used for sequencing the whole genome library and only 10 clones were randomly selected for analysis of the library sequences. However, high throughput sequencing of whole genome amplified product using RT-Bst SPA could be more useful for determining the whole genome sequences in this study. It is noteworthy that sequencing results obtained after random screening of clones from the whole genome library of hRSV and influenza B could be further improved if a higher number of clones could be selected for sequence analysis.

In this study, it was found that DNase digestion of the host genomic DNA from any sample is a key to success for WGA of virus genome from clinical samples. Different strategies have been employed by other workers for preferential isolation of virus particles and whole genome amplification. Stang *et al*. (2005) used low speed centrifugation to remove cellular debris and sucrose gradient ultracentrifugation to separate virus particles so that only virus sequences were amplified in random PCR. These strategies helped them to selectively amplify the sequences specific for the desired viruses. Stang *et al*., (2005) used larger amount

(11 ml of supernatant from cell culture) of sample which is not suitable for studying any nasopharyngeal sample that is usually collected in small volume. Nasopharyngeal samples are collected by touching the nasopharynx using a sterile swab and dipping it into a transport media. Allander *et al*., (2007) pooled ten respiratory samples and used 200 µl for extraction of nucleic acids. They also used filtration, ultracentrifugation, adapter ligation and SISPA techniques to recover virus sequences. However, it may not always be possible to pool multiple samples to obtain 200 µl of sample. The new approach RT-Bst PCR developed in this study requires relatively small amount of nasopharyngeal sample for WGA, starting from 10 µl to 50 µl and is therefore suitable in cases where the amount of clinical sample available is limiting. Subsequent sequence analysis suggested that appropriate DNase treatment was necessary to identify more positive sequences. Microfiltration was used by Allander *et al*., (2001) to sieve out host cells and organelles for purifying serum samples. In this study microfiltration was used for a minute amount (10 µl to 50 µl) of samples only but this treatment did not work well which could be due to the very low abundance of viruses in the sample (data not shown).

A major drawback of the PCR based WGA is that sequences that do not match with other sequences in the database cannot be classified (Stang *et al*., 2005). However, a cut off value such as, *E* <0.001 (Cann *et al*., 2005) or *E* <0.0001 was considered by other workers (Allander *et al*., 2007) to determine any significant similarity of the sequences when they used BLASTn tools (Section 2.8).

Other molecular biological techniques have also been used for the identification of new types of viruses such as low-stringency PCR for the detection of conserved regions of virus genome for discovery of SARS coronavirus (Drosten *et al*., 2003). Differential display was found useful for identification of human metapneumovirus and coronavirus (van den Hoogen *et al*., 2001; van der Hoek *et al*., 2004). Allander *et al*., (2007) discovered a third human polyomavirus using the DNase SISPA technique.

RT-Bst SPA, developed in the present study is simple and eliminated multiple steps involved in other techniques such as SISPA or particle-associated nucleic acid PCR for whole genome amplification. In this method viral RNA was amplified through RT-Bst amplification in one step followed by a single primer amplification to produce amplicons of different sizes. Although RT-Bst SPA is a similar approach to random PCR amplification from single stranded cDNA, it did not require a separate cDNA synthesis step. RT-Bst amplification not only converts RNA to cDNA but also enables the use of primers with tail sequences for subsequent PCR amplification (Section 6.2.4). Generally reverse transcription is a less efficient process and converts only 40-80% of RNA to cDNA depending on the type of random primers used (Stangegaard *et al*., 2006). In a preliminary phase of this study it was found that the cDNA concentration was improved after RT-Bst amplification when compared to reverse transcription alone (Section 4.4). Allander *et al*., (2001) discovered parvovirus, a DNA virus, using the SISPA technique with a sensitivity of 10^6 genome equivalent (GE) ml^{-1}. On the contrary RT-Bst was found to be useful for WGA of RNA viruses in this study. A summary of the different steps, the time spent and the cost involved in SISPA and RT-Bst techniques is shown in Table 6.5.

Table 6.5 Comparison of SISPA and RT-Bst SPA for whole genome amplification

WGA technique	SISPA			RT-Bst SPA		
Steps involved	**Requirement**	**Time**	**Cost**	**Requirement**	**Time**	**Cost**
Reverse transcription or synthesis of first strand	Yes	0.5 h	£ 3.0	Single tube reaction	2.5 h	£ 3.0
Second strand synthesis	Yes	2.0 h	£ 1.0			
Blunting double stranded cDNA	Yes	0.1 h	£ 0.5			
Column purification	Yes	0.5 h	£ 2.0			
Phosphorylation of 5' end of adapter	Yes	0.5 h	£ 1.0			
Column purification	Yes	0.5 h	£ 2.0			
Adapter ligation to double stranded cDNA	Yes	2.0 h	£ 2.0			
Column purification	Yes	0.5 h	£ 2.0			
PCR using single primer	Yes	3.0 h	£ 1.0	Yes	3.0 h	£ 1.0
Total time and cost required		9.6 h	£ 14.5		5.5 h	£ 4.0

In summary, RT-Bst SPA is a shorter and more cost effective technique compared to the SISPA technique. According to our calculation the RT-Bst SPA protocol took 4.1 hours less for amplification of the whole genome and required £10.50 less for reagent cost compared to the SISPA technique (Table 6.5). DNase treatment was an important step for successful whole genome amplification using RT-Bst SPA because of the differences in concentrations of host DNA in different samples. Unlike SISPA, RT-Bst SPA technique was a closed tube reaction and reduced the possibility of contamination during whole genome amplification. RT-Bst SPA would be a suitable, reliable, sensitive and cheaper option for whole genome amplification compared to SISPA provided an optimum DNase treatment was ascertained. This approach will allow possibilities for easy and low cost

amplification of whole genomes of RNA viruses. Amplified products can be used for different purposes for example, library preparation, next generation sequencing and microarray analysis. This technique can also be used for the amplification of other types of nucleic acid sequences.

Chapter 7

Application of HRM analysis for rapid detection of virus sequences

7.1 Introduction and aims

High resolution melting (HRM) of PCR products is a sensitive, rapid and economic method for the detection and characterization of nucleic acid sequences for mutation, polymorphism and epigenetic studies (Reed and Wittwer, 2004; De Leeneer *et al*., 2008; Takano *et al*., 2008). In conventional real-time PCR, melting curve analysis is performed to monitor the purity of amplified products using double stranded DNA (dsDNA) binding dyes or hybridization probes to monitor the amplification of desired sequences (Wittwer and Kusukawa, 2005). In HRM analysis DNA saturating dyes such as, EvaGreen are used which specifically bind to double stranded DNA (dsDNA) and emits fluorescence. The amount of fluorescence is proportional to the amount of DNA present in the sample. With the increase in temperature during the melting analysis dsDNA is denatured to single stranded DNA (ssDNA) and the DNA intercalating dye is released. Consequently, fluorescent intensity will gradually decrease to a basal level with the increase in the melting temperature. If the change in the fluorescence intensity is plotted against the transition in the temperature it produces a characteristic curve for individual DNA molecules based on their base composition and length which can be used for analysis of the sequence. With the development of HRM technology it is now possible to accurately control temperature transition and fluorescence data acquisition using sophisticated instruments during melting analysis (Gundry *et al*., 2003). The newest generation fluorescent DNA binding dyes for example, LC-Green and EvaGreen have improved saturation properties than the previously used SYBR Green I dye (Wittwer *et al*., 2003). These advancements in high resolution melting technology will help with accurate assessment of the sequence variations based on the melting behaviour of DNA without using expensive fluorescent probes for sequence analysis. Different real-time PCR instruments have been used for HRM analysis but they have been reported to vary in their performances for analysis of different sequences (Herrmann *et al*., 2006, 2007a, b).

PCR amplicons can be genotyped in a closed tube reaction using HRM analysis in the presence of a saturating DNA binding dye for example; EvaGreen and LC-

Green dyes (Wittwer *et al*., 2003). EvaGreen dye selectively binds to dsDNA and strongly fluoresces and makes DNA detection easier using a HRM instrument. Unlike SYBR Green I, EvaGreen dye can bind with equal affinity to GC-rich and AT-rich regions of DNA at higher concentrations which makes it a suitable dye for all types of PCR products. Amplicons are gradually denatured by raising the temperature of the reaction. The temperature at which 50% of the total DNA in the reaction is denatured is considered as the melting temperature (T_m). These characteristic melting patterns of different sequences can be used for analysis of different sequences in HRM assays. Melting temperature (T_m) can be estimated using the formula; T_m = 2°C x (number of [A+T]) + 4°C x (number of [G+C]). It is a function of the DNA GC-content (T_m is higher in GC-rich PCR products), length and sequence composition. The other factors which can interfere with melting curve analysis are quality of genomic DNA (gDNA), impurities in the extracted nucleic acids, primer design, dye selection and PCR reagents. The presence of salts in the extracted DNA will influence the thermodynamics of the DNA melting transition. The presence of any contaminants will complicate the interpretation of the DNA melt curve. Nonspecific PCR products can be produced from low-quality DNA leading to incorrect scoring of results in HRM analysis. Low copies of template can result in the late amplification of PCR products and low-resolution in HRM data (von Ahsen *et al*., 2001). Therefore, it is necessary to use the same reagents, buffers, protocols and nucleic acid extraction and purification processes for all of the samples including the controls to avoid the chances of variation in the experiment and analysis.

The fluorescence intensity decreases with the denaturation of the double stranded DNA and release of intercalating dye (LC Green). If the fluorescence signals are plotted against the increase in temperature it produces a unique curve from each type of sequence. The separation of the melt curves becomes more apparent after normalization of the pre-melt and post-melt fluorescence signals after the HRM analysis using a software for example; Rotor-Gene Q software 1.7. During this normalization process signal generated from all samples are set to a uniform

relative value within a range of 100% to 0%. Normalisation of the pre-melt and post-melt fluorescence data using the HRM analysis software brings similar sequences together and moves different sequences apart.

HRM analysis has been used for analysis of sequence variation in both eukaryotic and prokaryotic systems. Shorter sized amplicons showed better resolution of small sequence variation in a study of single nucleotide polymorphisms (SNPs) (Liew *et al*., 2004) and detection of pandemic influenza A H1N1 virus (Varillas *et al*., 2011) using HRM analysis. HRM analysis has also been found to be useful to distinguish between six common beta-globin genotypes using 110 bp amplicons whereas the hydroxytryptamine receptor 2A (HTR2A) single nucleotide polymorphism was genotyped in a 554 bp fragment (Wittwer *et al*., 2003). Other workers showed point mutation/deletion and qualitative nucleotide change in one assay using quantitative PCR and high resolution melting (qPCR-HRM) (Rouleau *et al*., 2009). Dobrowolski *et al*., (2009) analysed sequence variations in 16.6 kb human mitochondrial genome in less than 2 hours and demonstrated the sensitivity and speed of HRM analysis.

Provaznikova *et al*., (2008) reported that they could avoid more than 85% of the unnecessary sequencing of the myosin heavy chain (MYH) 9 gene when they used HRM analysis for pre-sequence screening. One of the limitations of HRM analysis is that it is usually not possible to apply this technique in multiplex mode to type variants in different fragments at the same time. However, Seipp *et al*., (2008) showed a quadruplex genotyping assay which is very sensitive and depends on the high quality of DNA and sensitivity of HRM system used. HRM has also been reported for assessment of DNA methylation patterns (Dahl and Guldberg, 2007). Epigenetic studies are important because DNA methylation patterns have been found to be associated with different diseases for example cancer and inherited disorders (Egger *et al*., 2004).

There are several other methods that are used to screen for mutation differences between two DNA sequences. These techniques include single-strand

conformational polymorphism (SSCP) (Shin *et al.*, 2010), denaturing gradient gel electrophoresis (DGGE) (Lerman and Silverstein, 1987), temperature gradient capillary electrophoresis (TGCE) (Li *et al*., 2002) and mass spectroscopy (Bocker, 2007). All of these methods require separation of samples either on gels or matrices and sometimes needs additional processing such as enzymatic or chemical reactions. Sequencing of the amplicons can provide more information on similarities and differences between sequences but can be expensive. With the development of DNA melting technology it is now possible to track minute changes in the melting behaviour of related sequences using HRM analysis (Gundry *et al.*, 2003). This technology has the potential to ascertain the sequence similarity by matching the melting pattern with a positive control. Appropriate use of HRM analysis will help reduce unnecessary costs for DNA sequencing. HRM analysis has been applied for identification and characterization of different microorganisms for example; mycobacterial typing using *hsp65* (Odell *et al*., 2005), identification of bacterial species by analysis of 16S rRNA genes (Cheng *et al*., 2006), detection of quinolone resistance (*gyrA* mutation) in *Salmonella* species (Slinger *et al*., 2007), detection of pandemic influenza A H1N1 virus (Varillas *et al*., 2011) and identification of *Aspergillus* species (Erali *et al*., 2006). However, there are only a limited number of publications available on the applications of HRM analysis for the detection and characterization of pathogenic viruses of the respiratory tract (Varillas *et al*., 2011).

One of the aims of this chapter was to demonstrate the application of HRM analysis (Erali *et al.*, 2008) for rapid and accurate detection of different types of sequences using a newly developed and sensitive instrument, the Rotor-Gene Q (QIAGEN, Crawley, UK). HRM analysis was used for pre-sequence screening from a whole genome library prepared from RNA virus genomes. The whole genome libraries were prepared from RNA viruses in this study using the RT-Bst SPA as described in chapter 6 (Figure 6.1). HRM analysis was also applied for the rapid and accurate detection of common respiratory tract virus sequences from cloned plasmids as an alternate to conventional sequence based detection.

7.2 Protocol for real-time PCR high resolution melting (RT-PCR-HRM)

7.2.1 Plasmid extraction and preparation of template

Three different libraries were prepared from MS2 RNA and RNA extracted from tissue culture media positive for hRSV and influenza B as described in chapter 6. Plasmids were extracted (Section 2.6.10) from ten randomly selected clones from each library for each type of virus. Plasmid concentrations were measured using a spectrophotometer (Section 2.6.2.1) and diluted to concentrations between 1-10 ng μl^{-1} to be used as templates for PCR followed by HRM (Section 7.2.2) using Type-iT™ HRM kit (QIAGEN, Crawley, UK).

7.2.2 Setting up PCR and HRM programmes using the Rotor-Gene Q

PCR reactions were set up using the Type-iT™ HRM kit (QIAGEN, Crawley, UK) according to table 7.1. A master mix was prepared and 8.0 µl was aliquoted in 0.1 ml PCR tubes. 2.0 µl of template was added to each tube in triplicate and placed in the real-time thermal cycler. Water was added to the negative control tubes instead of template to check for any nonspecific amplification or nucleic acid contamination in the reagents. Template DNA was added in sufficient amounts so that the cycle threshold (C_T) value for all samples fell below 30 and they did not differ by more than three C_T values. The cycle threshold (C_T) is defined as the number of cycles required for the fluorescence signals to exceed the background level of fluorescence. The fluorescence generated from the background can be determined from the fluorescence in the negative control.

Table 7.1: Reaction components using 2X HRM PCR master mix

Reaction component	Reaction volume (µl)	Final concentration
2X HRM PCR master mix (QIAGEN, Crawley, UK)	5.0	1X
Forward primer (10 µM)	0.7	0.7 µM
Reverse primer (10 µM)	0.7	0.7 µM
RNase-free water	1.6	--
Template DNA	2.0	1-20 ng plasmid per reaction
Total volume	10.0	--

The Rotor-Gene Q machine was set up according to Figures 7.1 and 7.2. The initial hold step was set at 95 ºC for 5 minutes to activate Hot Start Taq Plus DNA polymerase (not shown in Figure 7.1). The PCR cycling was set at 95 ºC for 10 seconds, 55 ºC for 30 seconds and 72 ºC for 10 seconds for 40 cycles following the manufacturer's instructions. Fluorescence data was acquired at the 72 ºC step through the green channel (source, 470 nm; detector, 510 nm). The High Resolution Melting protocol was followed as shown in figure 7.2. The temperature ramp (gradual increase in temperature to facilitate denaturation) was set at 5 ºC below the lowest T_m and 5 ºC above the highest T_m of all PCR products having a temperature rise by 0.1 ºC in each step following the manufacturer's instructions. Therefore, the melting temperature for the PCR products generated from the clones of MS2 and influenza B was set to ramp between 74 ºC and 94 ºC.

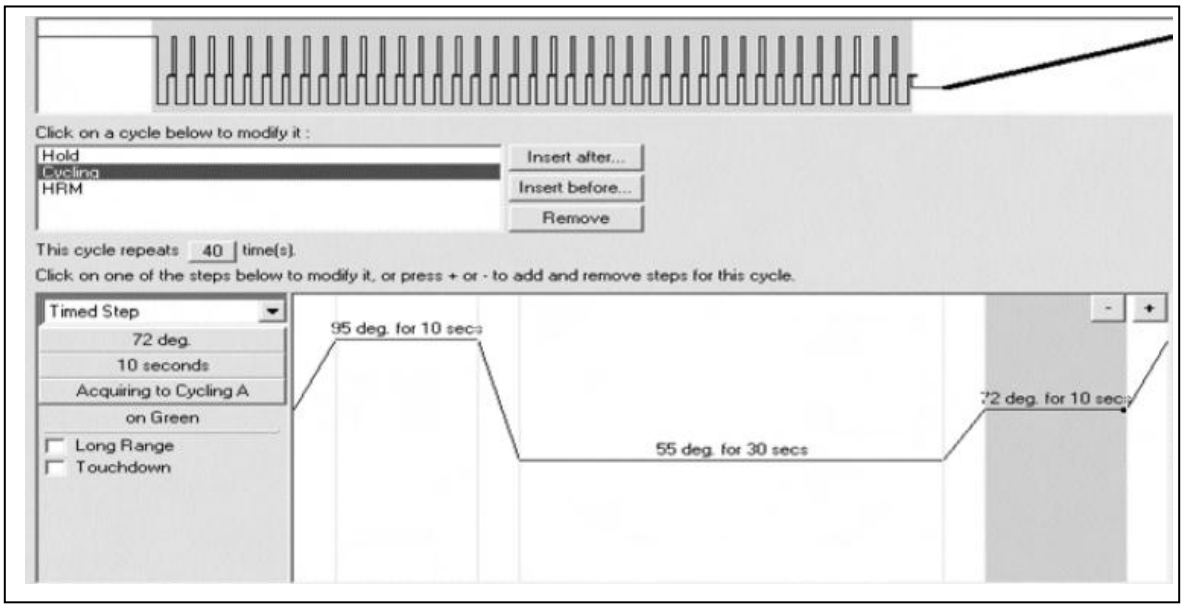

Figure 7.1: Cycling conditions for PCR using the Rotor-Gene system (Source: www.qiagen.com)

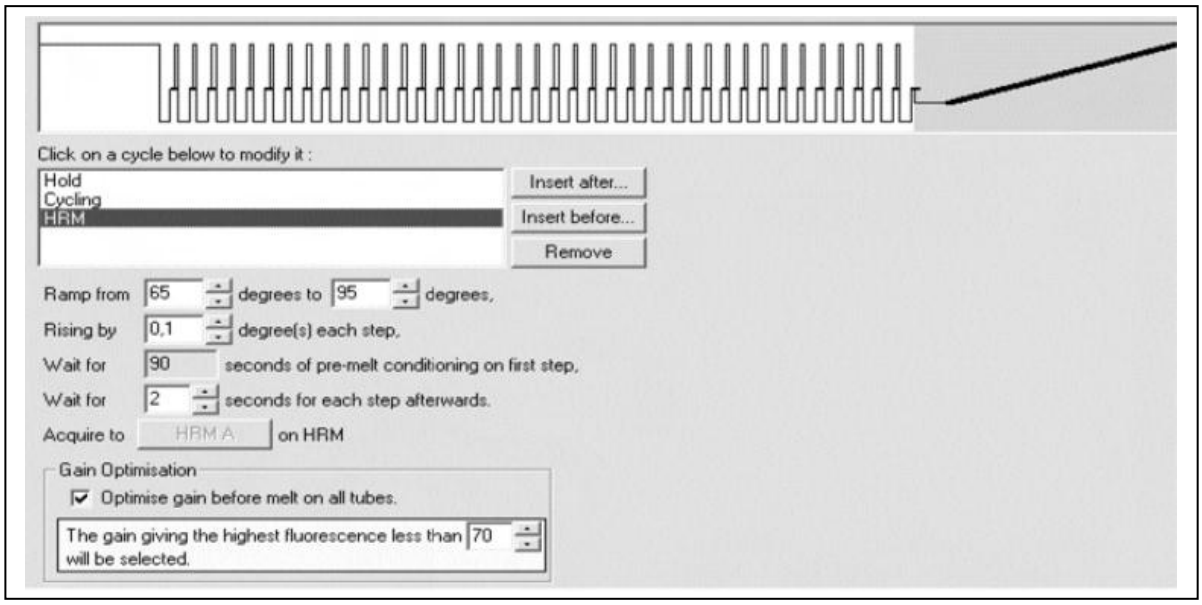

Figure 7.2: General configuration for HRM analysis using the Rotor-Gene system (Source: www.qiagen.com)

The ramp for the PCR products generated from the clones for hRSV was set between 73 ºC and 93 ºC. The ramp was determined from the melting behaviour of all of the sequences during optimisation of the HRM analysis. HRM data was analysed at the end of PCR and data acquisition using the Rotor-Gene Q software 1.7 (QIAGEN, Crawley, UK).

7.3 Pre-sequence screening of genomic libraries prepared from viral RNA by HRM analysis

Whole genome libraries were prepared from MS2 RNA genome and tissue culture media positive for hRSV and influenza B using the RT-Bst SPA (Chapter 6, Figure 6.1). However, screening a whole genome library is tedious, lengthy and expensive. Generally whole genome libraries are screened through selection of random clones and sequencing or immunoscreening using specific antibodies. In previous studies on sequence based analysis of the library sequences it has been found that most of the clones contained similar sequences (Provaznikova *et al*., 2008). Therefore, random screening of library sequences can be expensive and less productive. In this study we validated the application of HRM analysis for pre-sequence screening of genomic libraries to reduce expanses towards unnecessary sequencing which is described in figure 7.3 and in the following sections 7.3.1, 7.3.2 and 7.3.3.

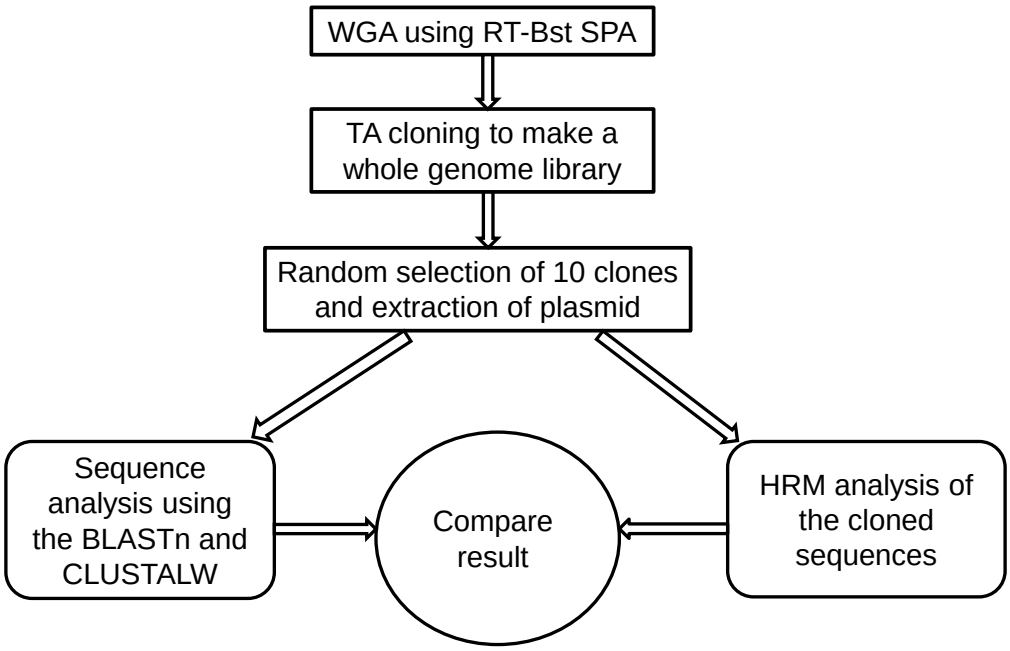

Figure 7.3: Presequence screening of genomic libraries prepared from viral RNA by HRM analysis (sequencing was done using either M13 F or R primer, PCR products were amplified using a single primer from the tail of the random primer, 5'-GACCATCTAGCGA CCTCCAC-3', before HRM analysis)

7.3.1 Pre-sequence screening of MS2 genomic library

A whole genome library was prepared from the MS2 RNA as described in the previous section 6.2. Clones were randomly selected from the genomic library to validate the HRM analysis as an alternate to conventional sequencing to determine the similarities and differences among the cloned sequences. Ten clones were randomly selected from the whole genome library of MS2 and the plasmids containing the inserts were sequenced (Section 2.6.10 and 2.7). Sequences corresponding to the 10 clones generated from the MS2 genomic library were compared using multiple sequence alignment tools CLUSTALW (http://www.ebi.ac.uk/Tools/msa/ clustalw2/). Sequences from clones 1, 2 and 3; clones 4, 5 and 10; clones 6, 7, 8 and 9 were found to fall into three different clusters after multiple sequence alignment (Figure 7.6, Table 6.4). After HRM

analysis and normalization of curves similar patterns for these three types of sequences were also found (Figure 7.5). Melting temperatures for these three groups (Table 7.2) were also slightly different which became evident after dissociation of the amplicons (Figure 7.5) and analysing the data using Rotor-Gene Q software 1.7. The average melting temperatures for sequences of clones 1, 2 and 3; clones 4, 5 and 10; clones 6, 7, 8 and 9 were 82.88 ºC; 82.91 ºC and 84.03 ºC, respectively (Table 7.2). It was found that similar sequences produced similar melting curves and *vice versa* (Figure 7.4). The similarities and differences between different sequences were clearly apparent from the normalized HRM curves (Figure 7.5).

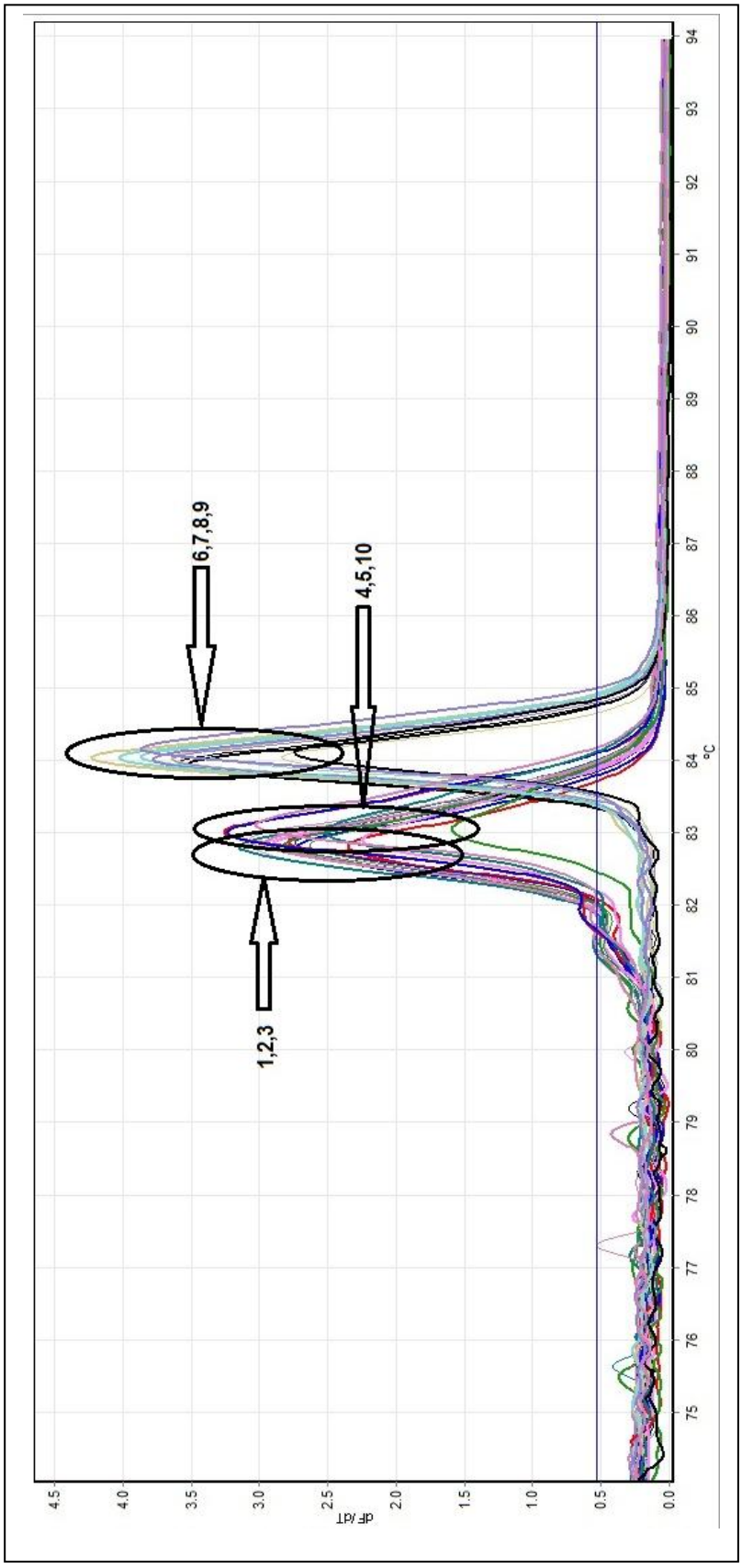

Figure 7.4: Melting analysis of the amplicons from triplicate samples of clones from MS2 in different colours. Clone numbers corresponding to the curves are shown with arrows. Colour codes are shown in table 7.2. X axis shows °C. Y axis shows change in fluorescence with time.

Colour	Name	Peak 1	Peak 2	Peak 3
	MS2-Clone-1	82.88		
	MS2-Clone-1	82.82		
	MS2-Clone-2	82.80		
	MS2-Clone-2	82.87		
	MS2-Clone-3	82.85		
	MS2-Clone-3	81.38	82.80	
	MS2-Clone-4	81.45	82.80	
	MS2-Clone-4	81.65	82.88	
	MS2-Clone-5	82.83		
	MS2-Clone-5	82.83		
	MS2-Clone-6	83.93		
	MS2-Clone-6	83.95		
	MS2-Clone-7	84.03		
	MS2-Clone-7	84.05		
	MS2-Clone-8	84.05		
	MS2-Clone-8	84.08		
	MS2-Clone-9	84.02		
	MS2-Clone-9	84.02		
	MS2-Clone-10	81.55	82.85	
	MS2-Clone-10	77.30	81.55	82.90
	MS2-Clone-1	83.02		
	MS2-Clone-2	81.95	83.00	
	MS2-Clone-3	82.90		
	MS2-Clone-4	83.02		
	MS2-Clone-5	83.05		
	MS2-Clone-6	84.10		
	MS2-Clone-7	84.03		
	MS2-Clone-8	84.03		
	MS2-Clone-9	84.15		
	MS2-Clone-10	81.85	83.10	

Table 7.2: Melting temperatures of different amplicons in triplicate for clones generated from MS2 RNA

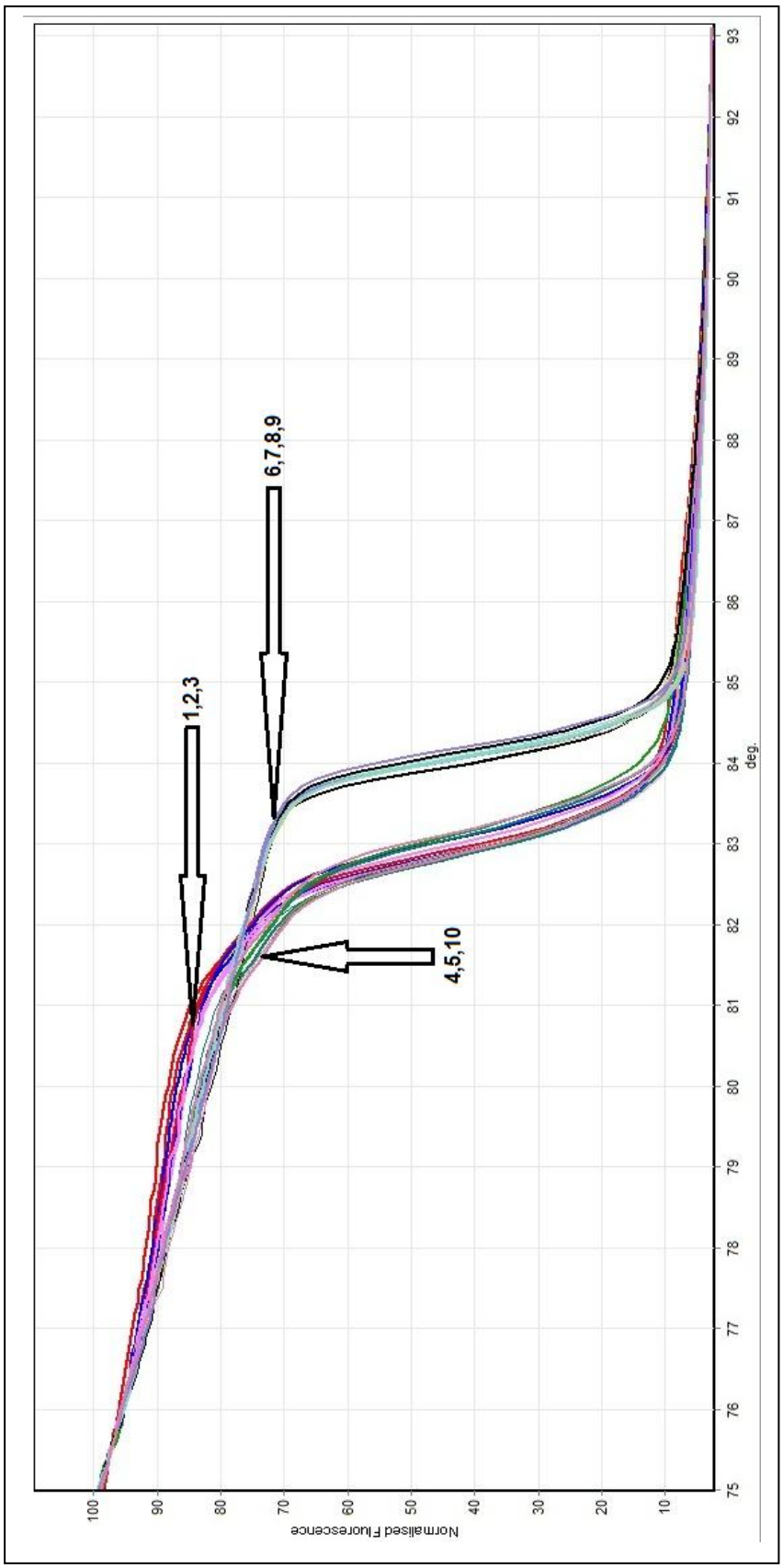

Figure 7.5: Normalized HRM curves generated from ten clones from whole genome library prepared from MS2 genome. Clone numbers corresponding to the curve are shown with arrows. X axis shows °C and Y axis shows normalized fluorescence. Colour codes are shown in table 7.3.

Figure 7.6: Alignment of the 10 clone sequences from the MS2 library using the CLUSTALW analysis software (http://www.ebi.ac.uk/Tools/services/webclustalw2/).

```
CLUSTAL 2.1 multiple sequence alignment for MS2 clone 1, 2 and 3

MS2-Clone-2-2592-2911-320bp      GACCATCTAGCGACCTCCACATAAAAGTGCGCGCCGCAGCTCTCGCGAAA 50
MS2-Clone-3-2590-2911-322bp      GACCATCTAGCGACCTCCACATAAAAGTGCGCGCCGCAGCTCTCGCGAAA 50
MS2-Clone-1-2592-2911-320bp      GACCATCTAGCGACCTCCACATAAAAGTGCGCGCCGCAGCTCTCGCGAAA 50
                                 **************************************************

MS2-Clone-2-2592-2911-320bp      GAGCCCGGACACGAACGTTTTACGAAGATTCGGTTTAAAACCGTAGTAGG 100
MS2-Clone-3-2590-2911-322bp      GAGCCCGGACACGAACGTTTTACGAAGATTCGGTTTAAAACCGTAGTAGG 100
MS2-Clone-1-2592-2911-320bp      GAGCCCGGACACGAACGTTTTACGAAGATTCGGTTTAAAACCGTAGTAGG 100
                                 **************************************************

MS2-Clone-2-2592-2911-320bp      CAAGTGCCTCTAGCACACGGGGTGCAATCTCACTGGGACATATAATATCG 150
MS2-Clone-3-2590-2911-322bp      CAAGTGCCTCTAGCACACGGGGTGCAATCTCACTGGGACATATAATATCG 150
MS2-Clone-1-2592-2911-320bp      CAAGTGCCTCTAGCACACGGGGTGCAATCTCACTGGGACATATAATATCG 150
                                 **************************************************

MS2-Clone-2-2592-2911-320bp      TCCCCGTAGATGCCTATGGTTCCGGCGTTACCAAAATGGATTTGGGTCGC 200
MS2-Clone-3-2590-2911-322bp      TCCCCGTAGATGCCTATGGTTCCGGCGTTACCAAAATGGATTTGGGTCGC 200
MS2-Clone-1-2592-2911-320bp      TCCCCGTAGATGCCTATGGTTCCGGCGTTACCAAAATGGATTTGGGTCGC 200
                                 **************************************************

MS2-Clone-2-2592-2911-320bp      TTTGACTATTGCCCAGAATATCATGGACTCTAGCTCAAATGTGAACCCAT 250
MS2-Clone-3-2590-2911-322bp      TTTGACTATTGCCCAGAATATCATGGACTCTAGCTCAAATGTGAACCCAT 250
MS2-Clone-1-2592-2911-320bp      TTTGACTATTGCCCAGAATATCATGGACTCTAGCTCAAATGTGAACCCAT 250
                                 **************************************************

MS2-Clone-2-2592-2911-320bp      TTCCCATTGTGGAAAATAGTTCCCATCGTATCGTCTCGCCATCTACGATT 300
MS2-Clone-3-2590-2911-322bp      TTCCCATTGTGGAAAATAGTTCCCATCGTATCGTCTCGCCATCTACGATT 300
MS2-Clone-1-2592-2911-320bp      TTCCCATTGTGGAAAATAGTTCCCATCGTATCGTCTCGCCATCTACGATT 300
                                 **************************************************

MS2-Clone-2-2592-2911-320bp      CCGTAGTGTGAGCGGATACGATCGAGATATGAATATAGCTCCGGTGGAGG 350
MS2-Clone-3-2590-2911-322bp      CCGTAGTGTGAGCGGATACGATCGAGATATGAATATAGCTCAGGTGGAGG 350
MS2-Clone-1-2592-2911-320bp      CCGTAGTGTGAGCGGATACGATCGAGATATGAATATAGCTC-GGTGGAGG 349
                                 ****************************************** ********

MS2-Clone-2-2592-2911-320bp      TCGCTAGATGGTC 363
MS2-Clone-3-2590-2911-322bp      TCGCTAGATGGTC 363
MS2-Clone-1-2592-2911-320bp      TCGCTAGATGGTC 362
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```
CLUSTAL 2.1 multiple sequence alignment for MS2 clones 4, 5 and 10


MS2-Clone-4-MS2sequence-2590-2      GACCATCTAGCGACCTCCACCTGAGCTATATTCATATCTCGATCGTATCC 50
MS2-Clone-10-MS2sequences-2590      GACCATCTAGCGACCTCCACCTGAGCTATATTCATATCTCGATCGTATCC 50
MS2-Clone-5-MS2sequence-2590-2      GACCATCTAGCGACCTCCACCTGAGCTATATTCATATCTCGATCGTATCC 50
                                    **************************************************

MS2-Clone-4-MS2sequence-2590-2      GCTCACACTACGGAATCGTAGATGGCGAGACGATACGATGGGAACTATTT 100
MS2-Clone-10-MS2sequences-2590      GCTCACACTACGGAATCGTAGATGGCGAGACGATACGATGGGAACTATTT 100
MS2-Clone-5-MS2sequence-2590-2      GCTCACACTACGGAATCGTAGATGGCGAGACGATACGATGGGAACTATTT 100
                                    **************************************************

MS2-Clone-4-MS2sequence-2590-2      TCCACAATGGGAAATGGGTTCACATTTGAGCTAGAGTCCATGATATTCTG 150
MS2-Clone-10-MS2sequences-2590      TCCACAATGGGAAATGGGTTCACATTTGAGCTAGAGTCCATGATATTCTG 150
MS2-Clone-5-MS2sequence-2590-2      TCCACAATGGGAAATGGGTTCACATTTGAGCTAGAGTCCATGATATTCTG 150
                                    **************************************************

MS2-Clone-4-MS2sequence-2590-2      GGCAATAGTCAAAGCGACCCAAATCCATTTTGGTAACGCCGGAACCATAG 200
MS2-Clone-10-MS2sequences-2590      GGCAATAGTCAAAGCGACCCAAATCCATTTTGGTAACGCCGGAACCATAG 200
MS2-Clone-5-MS2sequence-2590-2      GGCAATAGTCAAAGCGACCCAAATCCATTTTGGTAACGCCGGAACCATAG 200
                                    **************************************************

MS2-Clone-4-MS2sequence-2590-2      GCATCTACGGGGACGATATTATATGTCCCAGTGAGATTGCACCTCGTGTG 250
MS2-Clone-10-MS2sequences-2590      GCATCTACGGGGACGATATTATATGTCCCAGTGAGATTGCACCTCGTGTG 250
MS2-Clone-5-MS2sequence-2590-2      GCATCTACGGGGACGATATTATATGTCCCAGTGAGATTGCACCTCGTGTG 250
                                    **************************************************

MS2-Clone-4-MS2sequence-2590-2      CTAGAGGCACTTGCCTACTACGGTTTTAAACCGAATCTTCGTAAAACGTT 300
MS2-Clone-10-MS2sequences-2590      CTAGAGGCACTTGCCTACTACGGTTTTAAACCGAATCTTCGTAAAACGTT 300
MS2-Clone-5-MS2sequence-2590-2      CTAGAGGCACTTGCCTACTACGGTTTTAAACCGAATCTTCGTAAAACGTT 300
                                    **************************************************

MS2-Clone-4-MS2sequence-2590-2      CGTGTCCGGGCTCTTTCGCGAGAGCTGCGGCGCGCACTTTTACTGTGGTG 350
MS2-Clone-10-MS2sequences-2590      CGTGTCCGGGCTCTTTCGCGAGAGCTGCGGCGCGCACTTTTACTGTGGTG 350
MS2-Clone-5-MS2sequence-2590-2      CGTGTCCGGGCTCTTTCGCGAGAGCTGCGGCGCGCACTTTTACTGTGGTG 350
                                    **************************************************

MS2-Clone-4-MS2sequence-2590-2      TGGAGGTCGCTAGATGGTC 369
MS2-Clone-10-MS2sequences-2590      TGGAGGTCGCTAGATGGTC 369
MS2-Clone-5-MS2sequence-2590-2      TGGAGGTCGCTAGATGGTC 369
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CLUSTAL 2.1 multiple sequence alignment of MS2 clones 6, 7, 8 and 9


MS2-Clone-6-MS2sequences-1154-      GACCATCTAGCGACCTCCACCTACGCGAAGTTGCTTGGGGCGACAGTCAC 50
MS2-Clone-8-MS2sequences-1154-      GACCATCTAGCGACCTCCACCTACGCGAAGTTGCTTGGGGCGACAGTCAC 50
MS2-Clone-9-MS2sequences-1154-      GACCATCTAGCGACCTCCACCTACGCGAAGTTGCTTGGGGCGACAGTCAC 50
MS2-Clone-7-MS2sequences-1154-      ----------------------TACGCGAAGTTGCTTGGGGCGACAGTCAC 29
                                                           ***************************


MS2-Clone-6-MS2sequences-1154-      GTCG--AGTTCCGCCATTGTCGACGAGAACGAACTGAGTAGAGTTAGAAG 98
MS2-Clone-8-MS2sequences-1154-      GTCG--AGTTCCGCCATTGTCGACGAGAACGAACTGAGTAGAGTTAGAAG 98
MS2-Clone-9-MS2sequences-1154-      GTCGCCAGTTCCGCCATTGTCGACGAGAACGAACTGAGTAGAGTTAGAAG 100
MS2-Clone-7-MS2sequences-1154-      GTCGCCAGTTCCGCCATTGTCGACGAGAACGAACTGAGTAGAGTTAGAAG 79
                                    ****  *******************************************


MS2-Clone-6-MS2sequences-1154-      CCATGCTTCAAACTCCGGTTGAGGGCTCTATCTAGAGAGCCGTTGCCTGA 148
MS2-Clone-8-MS2sequences-1154-      CCATGCTTCAAACTCCGGTTGAGGGCTCTATCTAGAGAGCCGTTGCCTGA 148
MS2-Clone-9-MS2sequences-1154-      CCATGCTTCAAACTCCGGTTGAGGGCTCTATCTAGAGAGCCGTTGCCTGA 150
MS2-Clone-7-MS2sequences-1154-      CCATGCTTCAAACTCCGGTTGAGGGCTCTATCTAGAGAGCCGTTGCCTGA 129
                                    **************************************************


MS2-Clone-6-MS2sequences-1154-      TTAATGCTAACGCATCTAAGGTATGGACCATCGAGAAAGGGGACTTTACG 198
MS2-Clone-8-MS2sequences-1154-      TTAATGCTAACGCATCTAAGGTATGGACCATCGAGAAAGGGGACTTTACG 198
MS2-Clone-9-MS2sequences-1154-      TTAATGCTAACGCATCTAAGGTATGGACCATCGAGAAAGGGGACTTTACG 200
MS2-Clone-7-MS2sequences-1154-      TTAATGCTAACGCATCTAAGGTATGGACCATCGAGAAAGGGGACTTTACG 179
                                    **************************************************


MS2-Clone-6-MS2sequences-1154-      TACGCGCCAGTTGTTGGCCATACGGATTGTACCCCTCGATGGTGGAGGTC 248
MS2-Clone-8-MS2sequences-1154-      TACGCGCCAGTTGTTGGCCATACGGATTGTACCCCTCGATGGTGGAGGTC 248
MS2-Clone-9-MS2sequences-1154-      TACGCGCCAGTTGTTGGCCATACGGATTGTACCCCTCGATGGTGGAGGTC 250
MS2-Clone-7-MS2sequences-1154-      TACGCGCCAGTTGTTGGCCATACGGATTGTACCCCTCGATGGTGGAGGTC 229
                                    **************************************************


MS2-Clone-6-MS2sequences-1154-      GCTAGATGG---------------------- 257
MS2-Clone-8-MS2sequences-1154-      GCTAGATGGTC------------------- 259
MS2-Clone-9-MS2sequences-1154-      GCTAGATGGTC------------------- 261
MS2-Clone-7-MS2sequences-1154-      GCTAGATGGTCAAGGGCGAATTCCAGCACAC 260
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An * (asterisk) represents positions that have a single, fully conserved residue. A : (colon) represents conservation between groups of strongly similar properties. A . (period) is used for conservation between groups of weakly similar properties.

7.3.2 Application of HRM to screen genomic library prepared from tissue culture media positive for influenza B

A whole genome library was prepared from tissue culture media positive for influenza B as described in section 6.2. This whole genome library was used to validate the application of HRM analysis for characterization of the cloned sequences as an alternate to conventional sequencing. Ten clones were randomly picked from the whole genome library prepared from the tissue culture fluid positive for influenza B and the plasmids were sequenced (Section 2.6.10 and 2.7). The sequences generated from the 10 clones from the influenza B library were compared using multiple sequence alignment tools CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Sequences from clones 1 and 2; clones 3, 4 and 5; clones 6, 7 and 10 and clones 8 and 9 were found to fall into four different groups (Appendix vi, pages l-m) when they were analysed using the multiple sequence alignment tool CLUSTALW (Figure 7.9, Table 6.2). After HRM analysis and normalisation of the data generated from the sequences of the ten clones of influenza B, it was again found that similar sequences produced similar patterns of HRM melt curves (Figure 7.8). Figure 7.7 shows the conventional melting pattern of the different sequences from the ten clones of influenza B. It was found that similar sequences showed similar melting temperatures and they tended to group together when they were analysed using the Rotor-Gene Q software 1.7. The average melting temperatures for clones 1 and 2; clones 3, 4 and 5; clones 6, 7 and 10 and clones 8 and 9 were 82.41 ºC, 81.22 ºC, 85.56 ºC and 82.52 ºC, respectively (Table 7.3). HRM analyses was found useful for grouping similar sequences and differentiate between dissimilar sequences from the clones of influenza B.

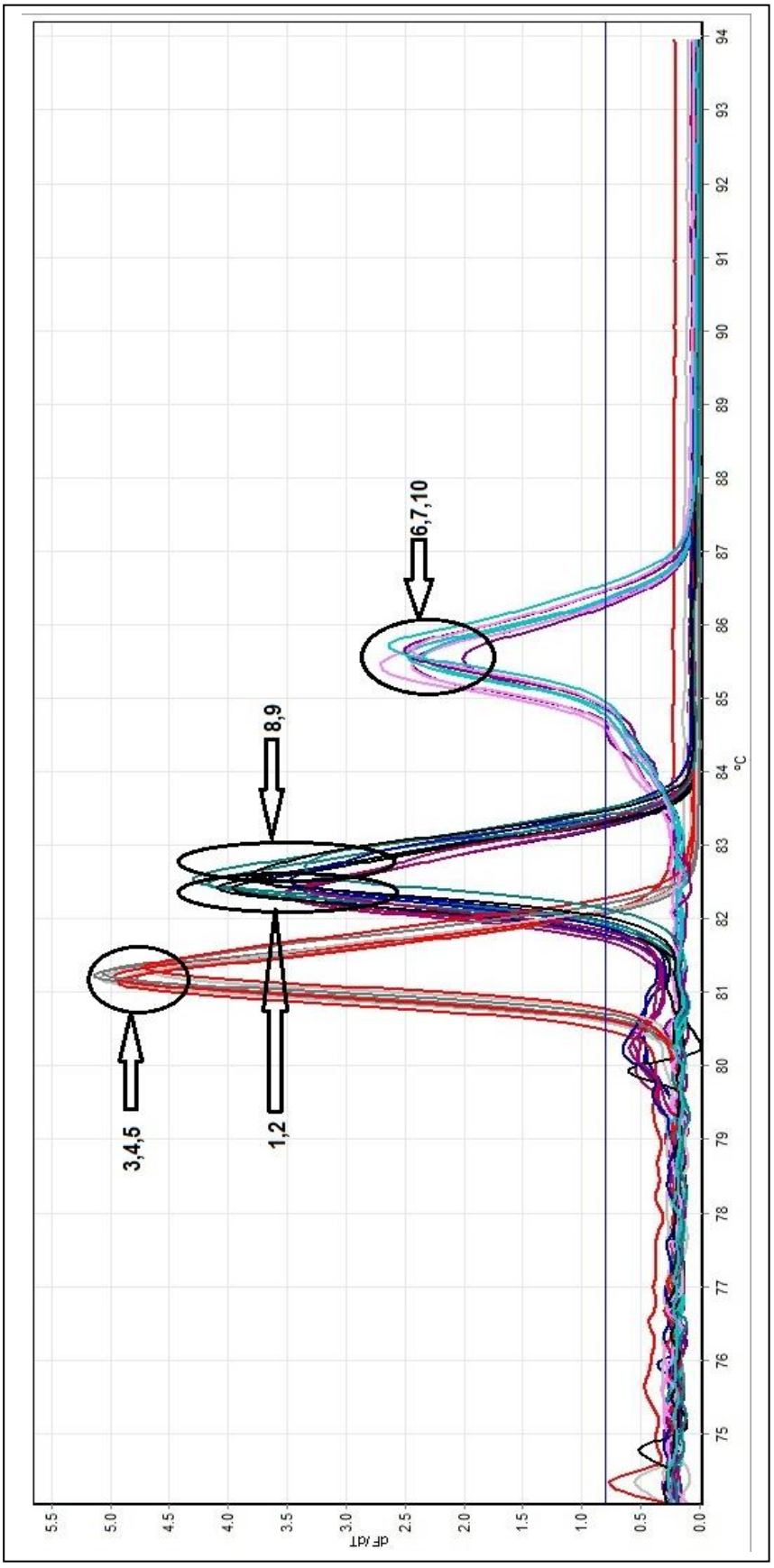

Figure 7.7: Melting patterns of the amplicons from ten clones from whole genome library prepared from influenza B. Clone numbers corresponding to the curves are shown with arrows. X axis shows °C and Y axis shows change in fluorescence with time dF/dT. Colour codes are shown in table 7.3.

Colour	Name	Peak 1
	Influenza B-Clone-1	82.52
	Influenza B-Clone-1	82.37
	Influenza B-Clone-2	82.33
	Influenza B-Clone-2	82.35
	Influenza B-Clone-3	81.17
	Influenza B-Clone-3	81.20
	Influenza B-Clone-4	81.20
	Influenza B-Clone-4	81.23
	Influenza B-Clone-5	81.15
	Influenza B-Clone-5	81.22
	Influenza B-Clone-6	85.53
	Influenza B Clone-6	85.47
	Influenza B-Clone-7	85.43
	Influenza B-Clone-7	85.45
	Influenza B-Clone-8	82.55
	Influenza B-Clone-8	82.48
	Influenza B-Clone-9	82.42
	Influenza B-Clone-9	82.42
	Influenza B-Clone-10	85.57
	Influenza B-Clone-10	85.58
	Influenza B-Clone-1	82.50
	Influenza B-Clone-2	82.43
	Influenza B-Clone-3	81.27
	Influenza B-Clone-4	81.25
	Influenza B-Clone-5	81.35
	Influenza B-Clone-6	85.65
	Influenza B-Clone-7	85.65
	Influenza B-Clone-8	82.70
	Influenza B-Clone-9	82.57
	Influenza B-Clone-10	85.72

Table 7.3: Melting temperatures for different clones of influenza B

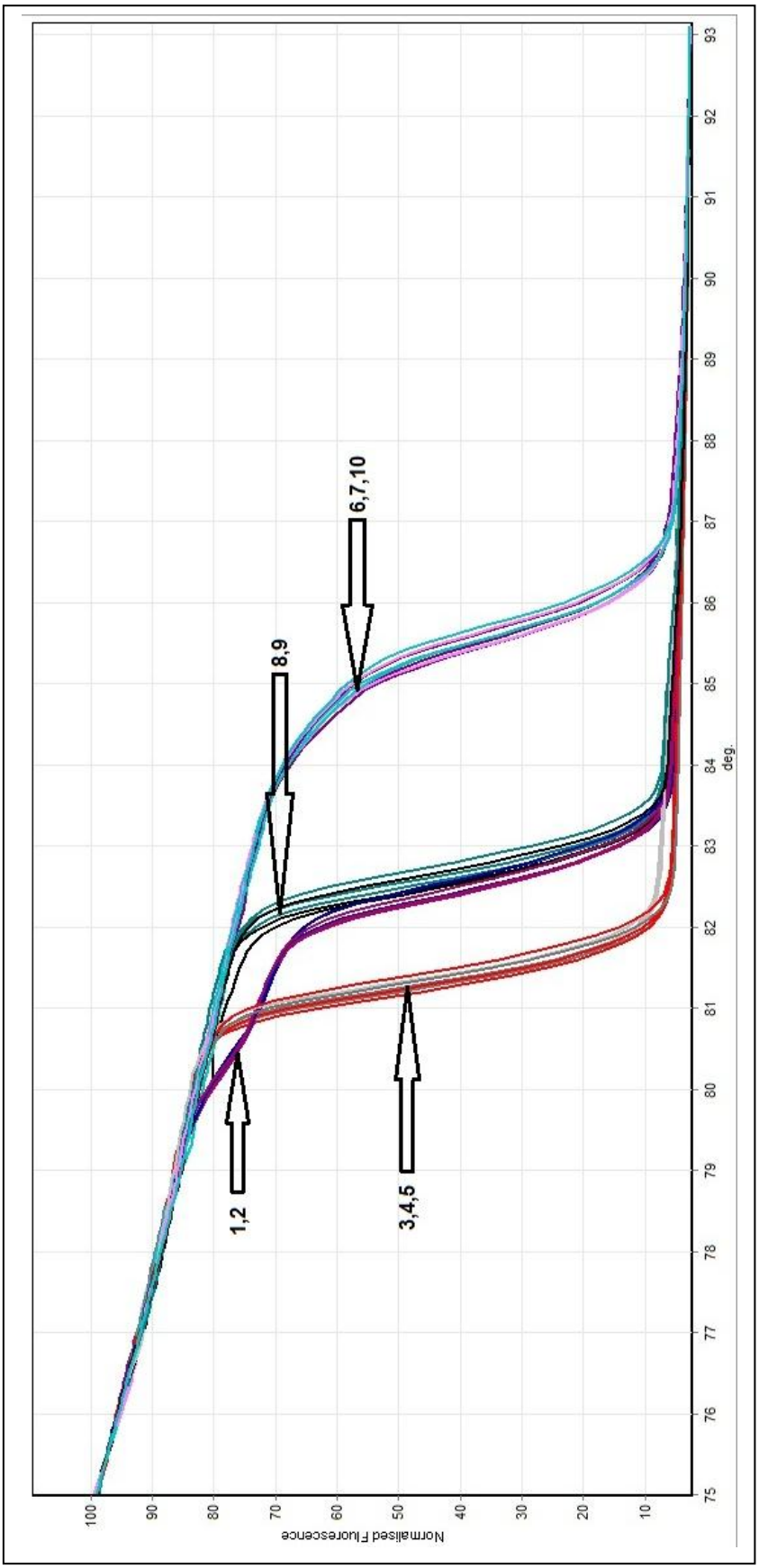

Figure 7.8: Normalized HRM curves generated from PCR amplicons of ten clones from whole genome library prepared from influenza B. Clone numbers corresponding to the curves are shown with arrows. X axis shows °C and Y axis shows normalized fluorescence. Colour codes are shown in table 7.3.

Figure 7.9: Multiple sequence alignment of the whole genome library sequences for tissue culture media positive for influenza B virus using the CLUSTALW analysis software (http://www.ebi.ac.uk/Tools/services/web clustalw2/)

```
CLUSTAL 2.1 multiple sequence alignment for influenza B clone 1 and 2


Influenza-B-Clone-1-InfB-Seg1-      GACCATCTAGCGACCTCCACAGCCCAGGGAGCGG-CTTGGAATAATCAGC 49
Influenza-B-Clone-2-InfB-Seg1-      GACCATCTAGCGACCTCCACAGCCCAGGGAGCGGGCTTGGAATAATCAGC 50
                                    ********************************** ***************

Influenza-B-Clone-1-InfB-Seg1-      GGGGAAAGAAGACCCTGTTGAGCTTGACTCTAGTCCGACTTGAAATGACT 99
Influenza-B-Clone-2-InfB-Seg1-      GGGGAAAGAAGACCCTGTTGAGCTTGACTCTAGTCCGACTTGAAATGACT 100
                                    ***************************************************

Influenza-B-Clone-1-InfB-Seg1-      TTCGAGATATAGCCTAAGTGGGAGCTAACGCACCG-TGTAAAACCACTGC 148
Influenza-B-Clone-2-InfB-Seg1-      TTCGAGATATAGCCTAAGTGGGAGCTAACGCACCGCTGTAAAACCACTGC 150
                                    ********************************** ***************

Influenza-B-Clone-1-InfB-Seg1-      TCGCTTAGCCATTTTACTTACTCCGTGAAGAAGTGGCCAATTTATTGGTT 198
Influenza-B-Clone-2-InfB-Seg1-      TCGCTTAGCCATTTTACTTACTCCGTGAAGAAGTGGCCAATTTATTGGTT 200
                                    ***************************************************

Influenza-B-Clone-1-InfB-Seg1-      CTTAGATTTAAACATCTATTTTATAGGTGTGAACTTTGCGGAGGACAAGG 248
Influenza-B-Clone-2-InfB-Seg1-      CTTAGATTTAAACATCTATTTTATAGGTGTGAACTTTGCGGAGGACAAGG 250
                                    ***************************************************

Influenza-B-Clone-1-InfB-Seg1-      TGAGGTGGAGGTCGCTAGATG--- 269
Influenza-B-Clone-2-InfB-Seg1-      TGAGGTGGAGGTCGCTAGATGGTC 274
                                    *********************
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CLUSTAL 2.1 multiple sequence alignment for influenza B clone 3,4 and 5


Influenza-B-Clone-3-Partially-      GACCATCTAGCGACCTCCACCCGCAACTTCTCCCATTGCTTCAAAAACTG 50
Influenza-B-Clone-5-NoMatch-In      GACCATCTAGCGACCTCCACCCGCAACTTCTCCCATTGCTTCAAAAACTG 50
Influenza-B-Clone-4-InfB-Seg-8      GACCATCTAGCGACCTCCACCCGCAACTTCTCCCATTGCTTCAAAAACTG 50
                                    ***************************************************

Influenza-B-Clone-3-Partially-      GAAAGTTGCTACCTGCGTTGGTATACATACTACCAACTGTTACCAAAACT 100
Influenza-B-Clone-5-NoMatch-In      GAAAGTTGCTACCTGCGTTGGTATACATACTACCAACTGTTACCAAAACT 100
Influenza-B-Clone-4-InfB-Seg-8      GAAAGTTGCTACCTGCGTTGGTATACATACTACCAACTGTTACCAAAACT 100
                                    ***************************************************

Influenza-B-Clone-3-Partially-      GCATAAGCATACCCTTGGTCAACGGATTGATACATTGTTTATAGAAAGCA 150
Influenza-B-Clone-5-NoMatch-In      GCATAAGCATACCCTTGGTCAACGGATTGATACATTGTTTATAGAAAGCA 150
Influenza-B-Clone-4-InfB-Seg-8      GCATAAGCATACCCTTGGTCAACGGATTGATACATTGTTTATAGAAAGCA 150
                                    ***************************************************

Influenza-B-Clone-3-Partially-      TTGAGGCTATTTCCATCGCAAGCTTTCTCTCTCGCGAGGAAAAGCATCCA 200
Influenza-B-Clone-5-NoMatch-In      TTGAGGCTATTTCCATCGCAAGCTTTCTCTCTCGCGAGGAAAAGCATCCA 200
Influenza-B-Clone-4-InfB-Seg-8      TTGAGGCTATTTCCATCGCAAGCTTTCTCTCTCGCGAGGAAAAGCATCCA 200
                                    ***************************************************

Influenza-B-Clone-3-Partially-      CATGTCCGCTTGGCAATACGAAAAGTGGATACCTTGCGTATACTCCTGAT 250
Influenza-B-Clone-5-NoMatch-In      CATGTCCGCTTGGCAATACGAAAAGTGGATACCTTGCGTATACTCCTGAT 250
Influenza-B-Clone-4-InfB-Seg-8      CATGTCCGCTTGGCAATACGAAAAGTGGATACCTTGCGTATACTCCTGAT 250
                                    ***************************************************

Influenza-B-Clone-3-Partially-      GGTGCTGTGGAGGTCGCTAGATGGTC 276
Influenza-B-Clone-5-NoMatch-In      GGTGCTGTGGAGGTCGCTAGATGGTC 276
Influenza-B-Clone-4-InfB-Seg-8      GGTGCTGTGGAGGTCGCTAGATGGTC 276
                                    *************************
```

```
CLUSTAL 2.1 multiple sequence alignment of influenza B clones 6, 7 and 10


Influenza-B-Clone-6-Partly-Euk      GACCATCTAGCGACCTCCACCCGCTCTGATTAATCCAGTTTTCACACCCA 50
Influenza-B-Clone-10-NoMatch        GACCATCTAGCGACCTCCACCCGCTCTGATTAATCCAGTTTTCACACCCA 50
Influenza-B-Clone-7-PartlyEuka      GACCATCTAGCGACCTCCACCCGCTCTGATTAATCCAGTTTTCACACCCA 50
                                    **************************************************

Influenza-B-Clone-6-Partly-Euk      ATGTCGGTCGATACGATCCAGTTACGCACATCTGGAGCGGACTCAATCTG 100
Influenza-B-Clone-10-NoMatch        ATGTCGGTCGATACGATCCAGTTACGCACATCTGGAGCGGACTCAATCTG 100
Influenza-B-Clone-7-PartlyEuka      ATGTCGGTCGATACGATCCAGTTACGCACATCTGGAGCGGACTCAATCTG 100
                                    **************************************************

Influenza-B-Clone-6-Partly-Euk      GCAAGTGAGCAAAGCATTTTCATGACGCTCACAGGCACAATCAACTCAAC 150
Influenza-B-Clone-10-NoMatch        GCAAGTGAGCAAAGCATTTTCATGACGCTCACAGGCACAATCAACTCAAC 150
Influenza-B-Clone-7-PartlyEuka      GCAAGTGAGCAAAGCATTTTCATGACGCTCACAGGCACAATCAACTCAAC 150
                                    **************************************************

Influenza-B-Clone-6-Partly-Euk      GACGACCGGTATTTTGCTGAACACGGTCAGCGTAAGGCCACCTGCGGACA 200
Influenza-B-Clone-10-NoMatch        GACGACCGGTATTTTGCTGAACACGGTCAGCGTAAGGCCACCTGCGGACA 200
Influenza-B-Clone-7-PartlyEuka      GACGACCGGTATTTTGCTGAACACGGTCAGCGTAAGGCCACCTGCGGACA 200
                                    **************************************************

Influenza-B-Clone-6-Partly-Euk      CCACCGACCCGAACGCATTGGATAACAGCGCTACCGACGCGACCTTGATC 250
Influenza-B-Clone-10-NoMatch        CCACCGACCCGAACGCATTGGATAACAGCGCTACCGACGCGACCTTGATC 250
Influenza-B-Clone-7-PartlyEuka      CCACCGACCCGAACGCATTGGATAACAGCGCTACCGACGCGACCTTGATC 250
                                    **************************************************

Influenza-B-Clone-6-Partly-Euk      GAAGCTCGGGCCTGTGGAGGTCGCTAGATGGTC 283
Influenza-B-Clone-10-NoMatch        GAAGCTCGGGCCTGTGGAGGTCGCTAGATGGTC 283
Influenza-B-Clone-7-PartlyEuka      GAAGCTCGGGCCTGTGGAGGTCGCTAGATGGTC 283
                                    *********************************
```

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CLUSTAL 2.1 multiple sequence alignment of influenza B clones 8 and 9


Influenza-B-Clone-8-PartlyEuka      GACCATCTAGCGACCTCCACCGTACAGAACCACCATTAGCACCATTACCG 50
Influenza-B-Clone-9-Partly-Euk      GACCATCTAGCGACCTCCACCGTACAGAACCACCATTAGCACCATTACCG 50
                                    **************************************************

Influenza-B-Clone-8-PartlyEuka      CCAGCATAATAAGTACCATAGCCGCCGCCCGTTGCAGTAATTGTATCAAA 100
Influenza-B-Clone-9-Partly-Euk      CCAGCATAATAAGTACCATAGCCGCCGCCCGTTGCAGTAATTGTATCAAA 100
                                    **************************************************

Influenza-B-Clone-8-PartlyEuka      AACAGAATTAGATCCCGATACCCCATTGTTGGTACCAGCCGCAGCACCGT 150
Influenza-B-Clone-9-Partly-Euk      AACAGAATTAGATCCCGATACCCCATTGTTGGTACCAGCCGCAGCACCGT 150
                                    **************************************************

Influenza-B-Clone-8-PartlyEuka      TTCCACCCGCACCAATAGTCACAGTAAATGATCCGGTTGAATAAGTAGCT 200
Influenza-B-Clone-9-Partly-Euk      TTCCACCCGCACCAATAGTCACAGTAAATGATCCGGTTGAATAAGTAGCT 200
                                    **************************************************

Influenza-B-Clone-8-PartlyEuka      CCTGGGGTTGTGGAGGTCGCTAGATGGTC 229
Influenza-B-Clone-9-Partly-Euk      CCTGGGGTTGTGGAGGTCGCTAGATGGTC 229
                                    *****************************
```

An * (asterisk) represents positions that have a single, fully conserved residue. A : (colon) represents conservation between groups of strongly similar properties. A . (period) is used for conservation between groups of weakly similar properties.

7.3.3 Application of the HRM analysis to screen a genomic library prepared from a tissue culture media positive for hRSV

A whole genome library was prepared from a tissue culture medium positive for hRSV as described in section 6.2 (Figure 6.1). Clones were randomly selected and screened using HRM analysis to validate its application as an alternative to conventional sequencing. As with the MS2 and the Influenza B libraries, ten clones were randomly selected from the whole genome library prepared for hRSV and the plasmids were sequenced after extraction (Section 2.6.1. and 2.7). Sequences generated from the 10 clones of the library prepared from a tissue culture medium positive for hRSV were compared using multiple sequence alignment tools CLUSTALW (http://www.ebi.ac.uk/ Tools/msa/clustalw2/). Ten sequences were found to fall into 5 different types after multiple sequence analysis using CLUSTALW tool (Figure 12). The sequence of clone 1 was different from all the other sequences. Clones 2 and 5; clones 3 and 4; clones 6, 7 and 8 and clones 9 and 10 were found to be similar based on the sequence information (Table 6.3). After HRM analysis and normalization of the HRM curves of clone 1; clones 2 and 5; clones 3 and 4; clones 6, 7 and 8 and clones 9 and 10 fell into five types of sequences (Figure 7.11). Figure 7.10 shows the conventional melting patterns of the different amplicons and they were found to group together when they had similar melting temperatures. After analysis of the data using Rotor-Gene Q software 1.7 it was found that the average melting temperatures for PCR products from clone 1; clones 3 and 4; clones 6, 7 and 8 and clones 9 and 10 were 78.92 ºC, 79.07 ºC, 81.27 ºC and 84.91 ºC, respectively (Table 7.4). However, each sequence from clone 2 and 5 produced two similar peaks at different temperatures of 83.18 ºC and 85.06 ºC. After comparing the sequence information and normalized HRM curves it was found that similar sequences produced similar melting patterns and *vice versa*.

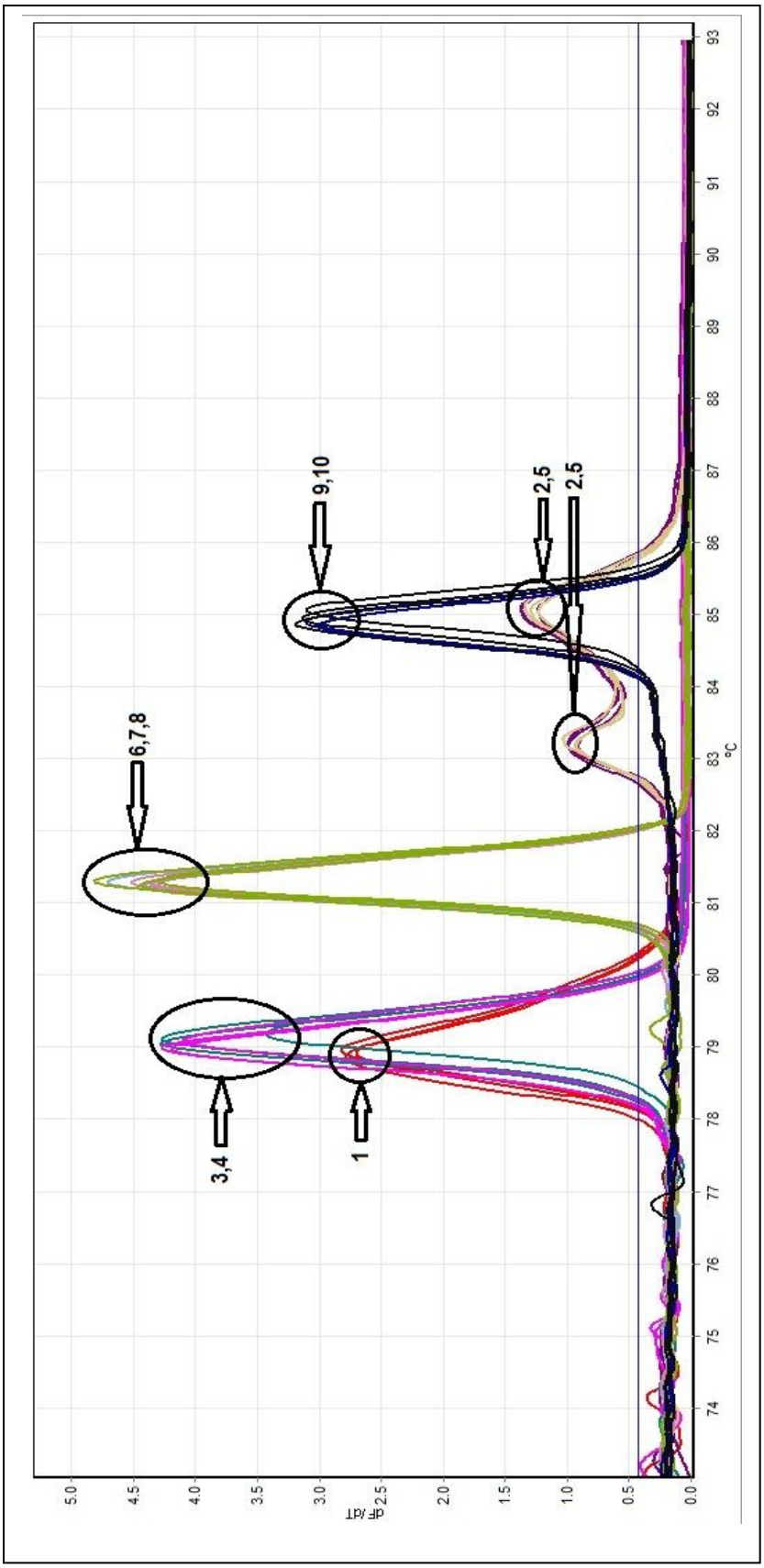

Figure 7.10: Melting patterns of the PCR amplicons from ten clones from whole genome library prepared from hRSV genome. Clone numbers corresponding to the curves are shown with arrows. Colour codes are shown in table 7.4. X axis shows °C and Y axis shows change in fluorescence with time.

Colour	Name	Peak 1	Peak 2
	hRSV-Clone-1	78.97	
	hRSV-Clone-1	78.92	
	hRSV-Clone-1	78.87	
	hRSV-Clone-2	83.18	85.05
	hRSV-Clone-2	83.18	85.05
	hRSV-Clone-2	83.20	85.07
	hRSV-Clone 3	79.12	
	hRSV-Clone 3	79.05	
	hRSV-Clone 3	79.20	
	hRSV-Clone 4	78.98	
	hRSV-Clone 4	79.00	
	hRSV-Clone 4	79.10	
	hRSV-Clone 5	83.18	85.05
	hRSV-Clone 5	83.25	85.10
	hRSV-Clone 5	83.20	85.05
	hRSV-Clone 6	81.30	
	hRSV-Clone 6	81.25	
	hRSV-Clone 6	81.25	
	hRSV-Clone 7	81.28	
	hRSV-Clone 7	81.23	
	hRSV-Clone 7	81.28	
	hRSV-Clone 8	81.30	
	hRSV-Clone 8	81.30	
	hRSV-Clone 8	81.25	
	hRSV-Clone 9	84.85	
	hRSV-Clone 9	84.90	
	hRSV-Clone 9	84.85	
	hRSV-Clone 10	84.87	
	hRSV-Clone 10	85.05	
	hRSV-Clone 10	84.95	

Table 7.4: Colour codes and melting temperatures for different clones of hRSV

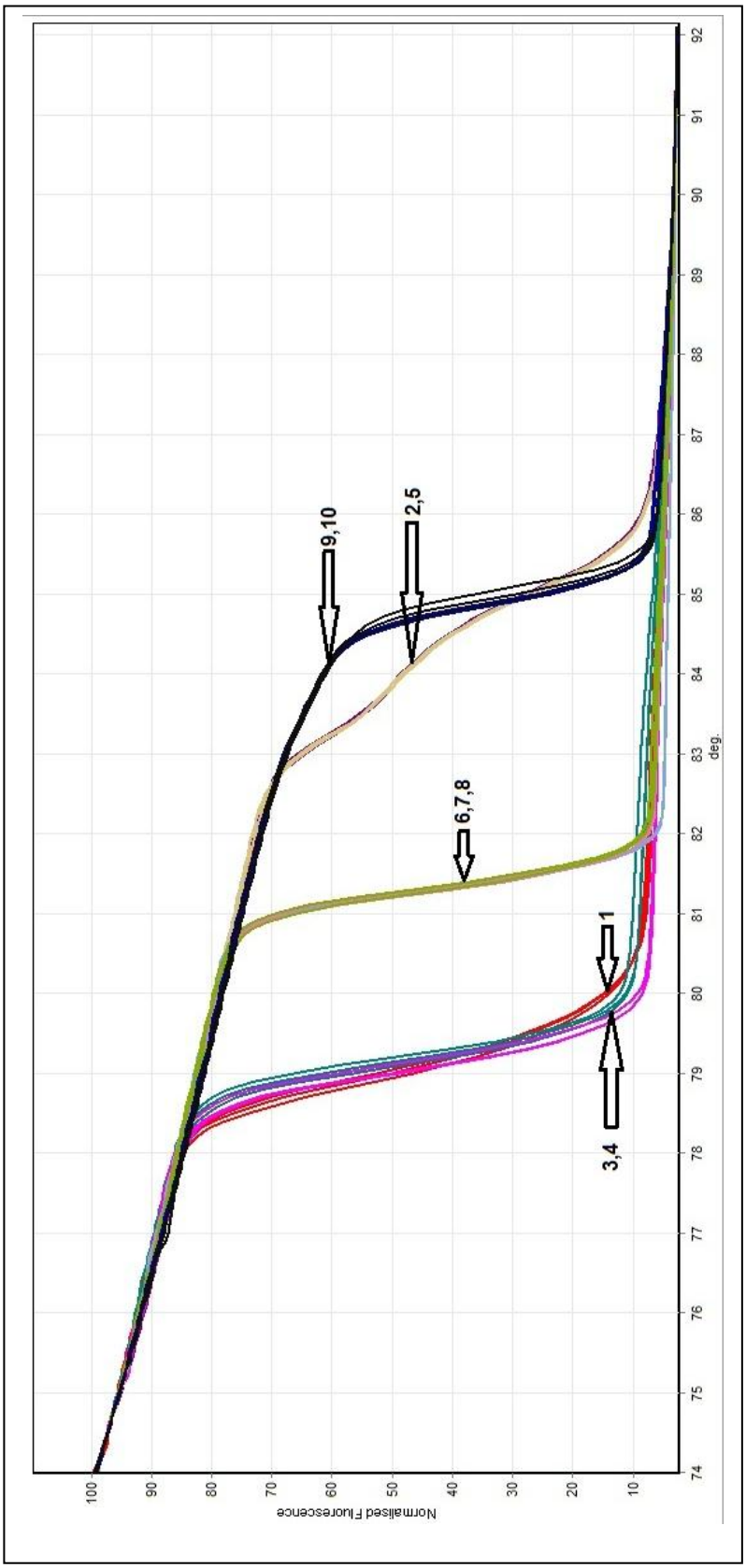

Figure 7.11: Normalized HRM analysis of the PCR amplicons from ten clones from the whole genome library prepared from hRSV genome. Clone numbers corresponding to the curves are shown with arrows. X axis shows °C and Y axis shows normalized fluorescence. Colour codes are shown in Table 7.4.

Figure 7.12: Multiple sequence alignment of the whole genome library sequences for tissue culture media positive for hRSV using the CLUSTALW analysis software (http://www.ebi.ac.uk/Tools/services/webclustalw2/)

```
CLUSTAL 2.1 multiple sequence alignment for hRSV clones 2 and 5


hRSV-Clone-2-HumanChro-17       GACCATCTAGCGACCTCCACCCACCAAGTTACAGAAGAGGATTAGCACCT 50
hRSV-Clone-5-HumanChro-17       GACCATCTAGCGACCTCCACCCACCAAGTTACAGAAGAGGATTAGCACCT 50
                                **************************************************

hRSV-Clone-2-HumanChro-17       TGCTCTCACTGTGGAGAGAGAGGGAGAATCGCTGGCCCTCACGGGCTGCC 100
hRSV-Clone-5-HumanChro-17       TGCTCTCACTGTGGAGAGAGAGGGAGAATCGCTGGCCCTCACGGGCTGCC 100
                                **************************************************

hRSV-Clone-2-HumanChro-17       AGGCGTGGGGAGGCCGACAGACCCAGTGGATGAGCTAGAAGGGAGAGAAT 150
hRSV-Clone-5-HumanChro-17       AGGCGTGGGGAGGCCGACAGACCCAGTGGATGAGCTAGAAGGGAGAGAAT 150
                                **************************************************

hRSV-Clone-2-HumanChro-17       GACATTTGTTCATATTCCTAAAGGAGCTGCTTTCCTGGGTCAGAGCCTTA 200
hRSV-Clone-5-HumanChro-17       GACATTTGTTCATATTCCTAAAGGAGCTGCTTTCCTGGGTCAGAGCCTTA 200
                                **************************************************

hRSV-Clone-2-HumanChro-17       GCTTCTCTAGTCCCTCTTTCTCTTTGAGCATCTATTAACCACCAATGGGG 250
hRSV-Clone-5-HumanChro-17       GCTTCTCTAGTCCCTCTTTCTCTTTGAGCATCTATTAACCACCAATGGGG 250
                                **************************************************

hRSV-Clone-2-HumanChro-17       GGCCACTAGAGGGCCTGTGGAGGTCGCTAGATGGTC 286
hRSV-Clone-5-HumanChro-17       GGCCACTAGAGGGCCTGTGGAGGTCGCTAGATGGTC 286
                                ************************************
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CLUSTAL 2.1 multiple sequence alignment hRSV clones 3 and 4


hRSV-Clone-3-HumanChro-17       GACCATCTAGCGACCTCCACATGCGAAACACATTGTAGGAGCTCAGAAGA 50
hRSV-Clone-4-HumanChro-17       GACCATCTAGCGACCTCCACATGCGAAACACATTGTAGGAGCTCAGAAGA 50
                                **************************************************

hRSV-Clone-3-HumanChro-17       TGTCAGCAATAGTTTTTATCTTACCACTATCACTAATGTTACTACCATTG 100
hRSV-Clone-4-HumanChro-17       TGTCAGCAATAGTTTTTATCTTACCACTATCACTAATGTTACTACCATTG 100
                                **************************************************

hRSV-Clone-3-HumanChro-17       ATGTTATTCTCATCATCATTATAATTATCTCTGAGTGCTCAGCATCTAGT 150
hRSV-Clone-4-HumanChro-17       ATGTTATTCTCATCATCATTATAATTATCTCTGAGTGCTCAGCATCTAGT 150
                                **************************************************

hRSV-Clone-3-HumanChro-17       AGATGCTTGGTAAATATCAAATCAGTGGATGAATGACTACCTCTAGAAGC 200
hRSV-Clone-4-HumanChro-17       AGATGCTTGGTAAATATCAAATCAGTGGATGAATGACTACCTCTAGAAGC 200
                                **************************************************

hRSV-Clone-3-HumanChro-17       TCCTTTTATTCCTTCATTACAGGTCCTGGCTCCCTGACTTCATTTCTCCT 250
hRSV-Clone-4-HumanChro-17       TCCTTTTATTCCTTCATTACAGGTCCTGGCTCCCTGACTTCATTTCTCCT 250
                                **************************************************

hRSV-Clone-3-HumanChro-17       ATATATTTTATGCAGGATCTGCTTCATGGCAGAACAGAGTAATGGTTAAG 300
hRSV-Clone-4-HumanChro-17       ATATATTTTATGCAGGATCTGCTTCATGGCAGAACAGAGTAATGGTTAAG 300
                                **************************************************

hRSV-Clone-3-HumanChro-17       AGCTGGGGTGGAGGTCGCTAGATGGTC 327
hRSV-Clone-4-HumanChro-17       AGCTGGGGTGGAGGTCGCTAGATGGTC 327
                                ***************************
```

```
CLUSTAL 2.1 multiple sequence alignment for hRSV clones 6, 7 and 8


hRSV-Clone-6-Partially-Matched      GACCATCTAGCGACCTCCACCGCCCTTAATCCATTTAACCCTGAGTTGAC 50
hRSV-Clone-8-PartMatched-hRSV-      GACCATCTAGCGACCTCCACCGCCCTTAATCCATTTAACCCTGAGTTGAC 50
hRSV-Clone7-PartMatched-hRSV-1      GACCATCTAGCGACCTCCACCGCCCTTAATCCATTTAACCCTGAGTTGAC 50
                                    **************************************************


hRSV-Clone-6-Partially-Matched      ACAGCACATGTTTCAGAGAGCACGGGCTTGGGGGTAAGGTTATAGATTAA 100
hRSV-Clone-8-PartMatched-hRSV-      ACAGCACATGTTTCAGAGAGCACGGGCTTGGGGGTAAGGTTATAGATTAA 100
hRSV-Clone7-PartMatched-hRSV-1      ACAGCACATGTTTCAGAGAGCACGGGCTTGGGGGTAAGGTTATAGATTAA 100
                                    **************************************************


hRSV-Clone-6-Partially-Matched      CAGCATCCCAAGGCAGAAAAATTTTTCGTAGTACAGAACAAAATGGAGTC 150
hRSV-Clone-8-PartMatched-hRSV-      CAGCATCCCAAGGCAGAAAAATTTTTCGTAGTACAGAACAAAATGGAGTC 150
hRSV-Clone7-PartMatched-hRSV-1      CAGCATCCCAAGGCAGAAAAATTTTTCGTAGTACAGAACAAAATGGAGTC 150
                                    **************************************************


hRSV-Clone-6-Partially-Matched      TCCTATGTTTACTTCTTTCTACCCAGACACAGTAACAATCTGATCTCTCT 200
hRSV-Clone-8-PartMatched-hRSV-      TCCTATGTTTACTTCTTTCTACCCAGACACAGTAACAATCTGATCTCTCT 200
hRSV-Clone7-PartMatched-hRSV-1      TCCTATGTTTACTTCTTTCTACCCAGACACAGTAACAATCTGATCTCTCT 200
                                    **************************************************


hRSV-Clone-6-Partially-Matched      GTCTTTTCCCCACATTCCCCCTTTTCTTTTCGACAAAACCGCCATTGTCA 250
hRSV-Clone-8-PartMatched-hRSV-      GTCTTTTCCCCACATTCCCCCTTTTCTTTTCGACAAAACCGCCATTGTCA 250
hRSV-Clone7-PartMatched-hRSV-1      GTCTTTTCCCCACATTCCCCCTTTTCTTTTCGACAAAACCGCCATTGTCA 250
                                    **************************************************


hRSV-Clone-6-Partially-Matched      TCATGGCCCATTCTCGATGGTCGCTTTCTCTTGTGGAGGTCGCTAGATGG 300
hRSV-Clone-8-PartMatched-hRSV-      TCATGGCCCATTCTCGATGGTCGCTTTCTCTTGTGGAGGTCGCTAGATGG 300
hRSV-Clone7-PartMatched-hRSV-1      TCATGGCCCATTCTCGATGGTCGCTTTCTCTTGTGGAGGTCGCTAGATGG 300
                                    **************************************************


hRSV-Clone-6-Partially-Matched      TC 302
hRSV-Clone-8-PartMatched-hRSV-      TC 302
hRSV-Clone7-PartMatched-hRSV-1      TC 302
                                    **
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CLUSTAL 2.1 multiple sequence alignment for hRSV clones 9 and 10


hRSV-Clone-9-HumanChrom8-Full       GACCATCTAGCGACCTCCACCAGACAAAAACAGGACCGCACGATGGAATG 50
hRSV-Clone-10-HumanCrhom-8-Ful      GACCATCTAGCGACCTCCACCAGACAAAAACAGGACCGCACGATGGAATG 50
                                    **************************************************


hRSV-Clone-9-HumanChrom8-Full       AGAGAGCTGCAGTGACAGGGGGACGTTGAGGCAGGCAGAGGTGAGAAGCA 100
hRSV-Clone-10-HumanCrhom-8-Ful      AGAGAGCTGCAGTGACAGGGGGACGTTGAGGCAGGCAGAGGTGAGAAGCA 100
                                    **************************************************


hRSV-Clone-9-HumanChrom8-Full       GTGAGAACGAAGGGAATGCTGGGTGCATCCTGAGGAAGCCTCTCCCATCT 150
hRSV-Clone-10-HumanCrhom-8-Ful      GTGAGAACGAAGGGAATGCTGGGTGCATCCTGAGGAAGCCTCTCCCATCT 150
                                    **************************************************


hRSV-Clone-9-HumanChrom8-Full       AGAAGCCACCAGGACTATCGCAGGCGGGCTATGAAACACCAGGCTTTGGA 200
hRSV-Clone-10-HumanCrhom-8-Ful      AGAAGCCACCAGGACTATCGCAGGCGGGCTATGAAACACCAGGCTTTGGA 200
                                    **************************************************


hRSV-Clone-9-HumanChrom8-Full       GCAAGATGACTGTGTCTCTCCCAAAAGGCTTACAGAAGCAAAATTGAGTT 250
hRSV-Clone-10-HumanCrhom-8-Ful      GCAAGATGACTGTGTCTCTCCCAAAAGGCTTACAGAAGCAAAATTGAGTT 250
                                    **************************************************


hRSV-Clone-9-HumanChrom8-Full       CGTGTGGAGGTCGCTAGATGGTC 273
hRSV-Clone-10-HumanCrhom-8-Ful      CGTGTGGAGGTCGCTAGATGGTC 273
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An * (asterisk) represents positions that have a single, fully conserved residue. A : (colon) represents conservation between groups of strongly similar properties. A . (period) is used for conservation between groups of weakly similar properties.

7.4 Application of HRM for rapid detection of common pathogenic viruses of the respiratory tract

PCR products positive for influenza B, hRSV, hRV and parainfluenza 2 were cloned in vector pCR2.1 as described in section 2.6.8. These vectors were transformed into chemically competent cells (*E coli* XL1-Blue, Agilent Technology, Edinburgh, UK) and propagated in Luria Bertani broth containing kanamycin (30 µg μl^{-1}) (Section 2.6.8). Plasmids were extracted (Section 2.6.10) from these clones and used as templates after quantitation and appropriate dilution as described in section 7.2. Commercially available plasmid (H1N1 Universal Control Plasmid V3, 2473 bp) containing the sequences for influenza A and swine influenza A was obtained from Integrated DNA Technology (IDT), Leuven, Belgium to be used as positive control for the respective viruses. Details of the primers used for influenza B, hRSV, hRV and parainfluenza 2 are provided in Table 2.4. Primers designed by WHO for the detection of influenza A and swine influenza A were obtained from IDT, Leuven, Belgium (Appendices i, Table A1). The PCR protocol and analysis was done according to the protocol mentioned in section 7.2 with some modifications. The PCR cycle was optimized for all of the viruses as follows: 95 ºC for 10 seconds, 55 ºC for 30 seconds and 72 ºC for 30 seconds for 40 cycles. The melting temperature for the PCR products generated from the viruses were set to ramp (gradual increase in melting temperature) between 72 ºC and 89 ºC. The average melting temperatures for influenza B, hRSV, hRV and influenza A were 79.48 °C, 78.59 °C, 80.34 °C and 80.58 °C, respectively (Table 7.5). Two distinct peaks were observed for the melting curves for parainfluenza 2 at 79.07 °C and 81.27 °C. Two peaks were also observed in the melting curves of swine influenza A at 77.42 °C and 77.83 °C (Table 7.5). All of the selected viruses showed distinct melting temperatures and HRM melting patterns for different amplicons when they were analysed using Rotor-Gene Q software 1.7 (Figure 7.13 and 7.14).

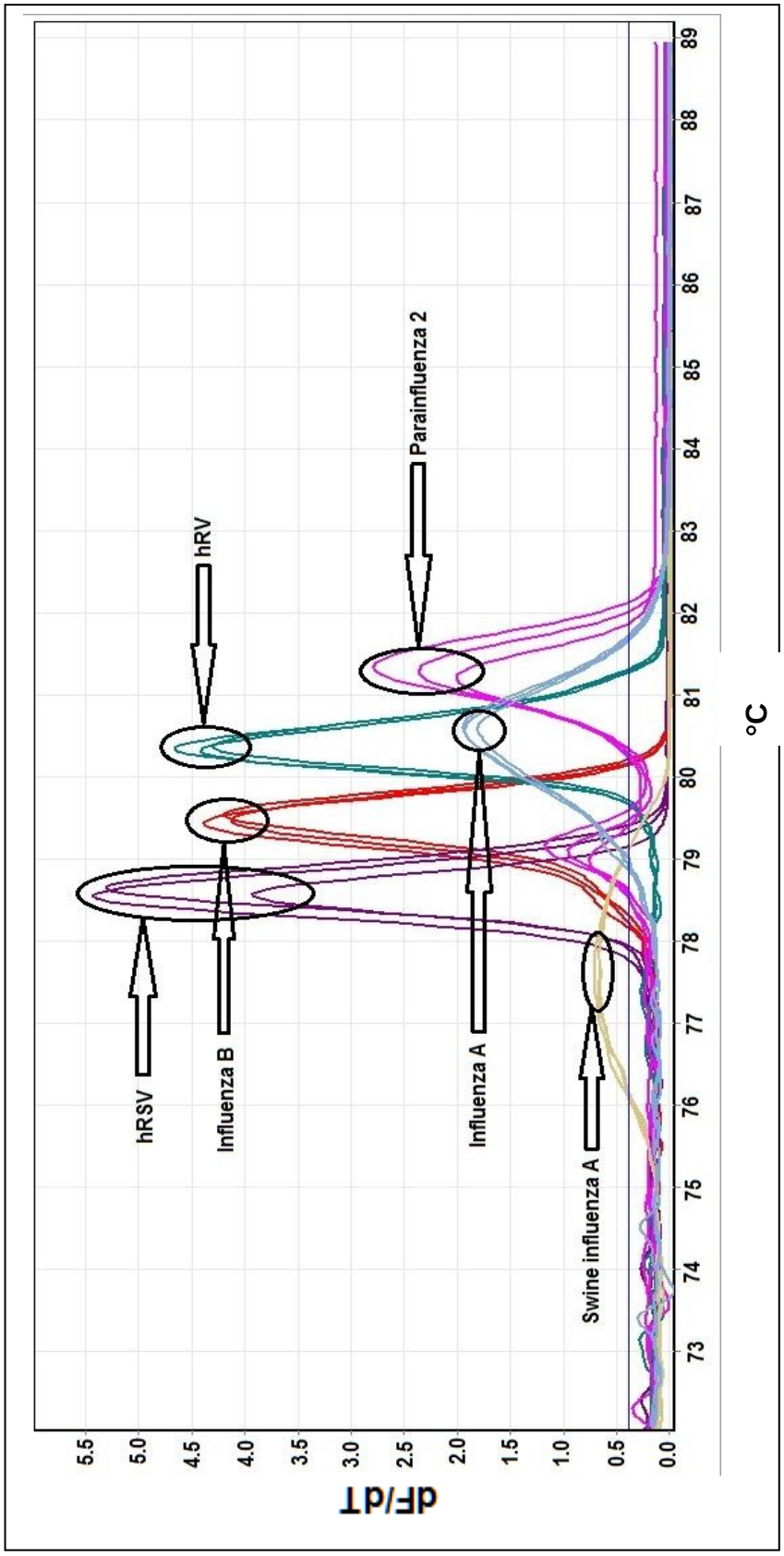

Figure 7.13: Melting analysis of the amplicons from known virus sequences of hRSV, influenza B, influenza A, swine influenza A, hRV and parainfluenza 2. Viruses corresponding to the curves are shown with arrows. Colour codes are shown in table 7.5. X axis shows °C and Y axis shows changes in fluorescence with time.

Colour	Name	Peak 1	Peak 2
	Influenza B	79.47	
	Influenza B	79.43	
	Influenza B	79.55	
	N35 hRSV	78.65	
	N35 hRSV	78.57	
	N35 hRSV	78.55	
	N15 hRV	80.35	
	N15 hRV	80.32	
	N15 hRV	80.37	
	Parainfluenza 2	79.15	81.33
	Parainfluenza 2	79.10	81.30
	Parainfluenza 2	78.98	81.20
	Swine influenza A	77.45	77.85
	Swine influenza A	77.45	77.80
	Swine influenza A	77.37	77.85
	Influenza A	80.58	
	Influenza A	80.57	
	Influenza A	80.60	

Table 7.5: Colour codes and melting temperatures for common pathogenic viruses of the respiratory tract

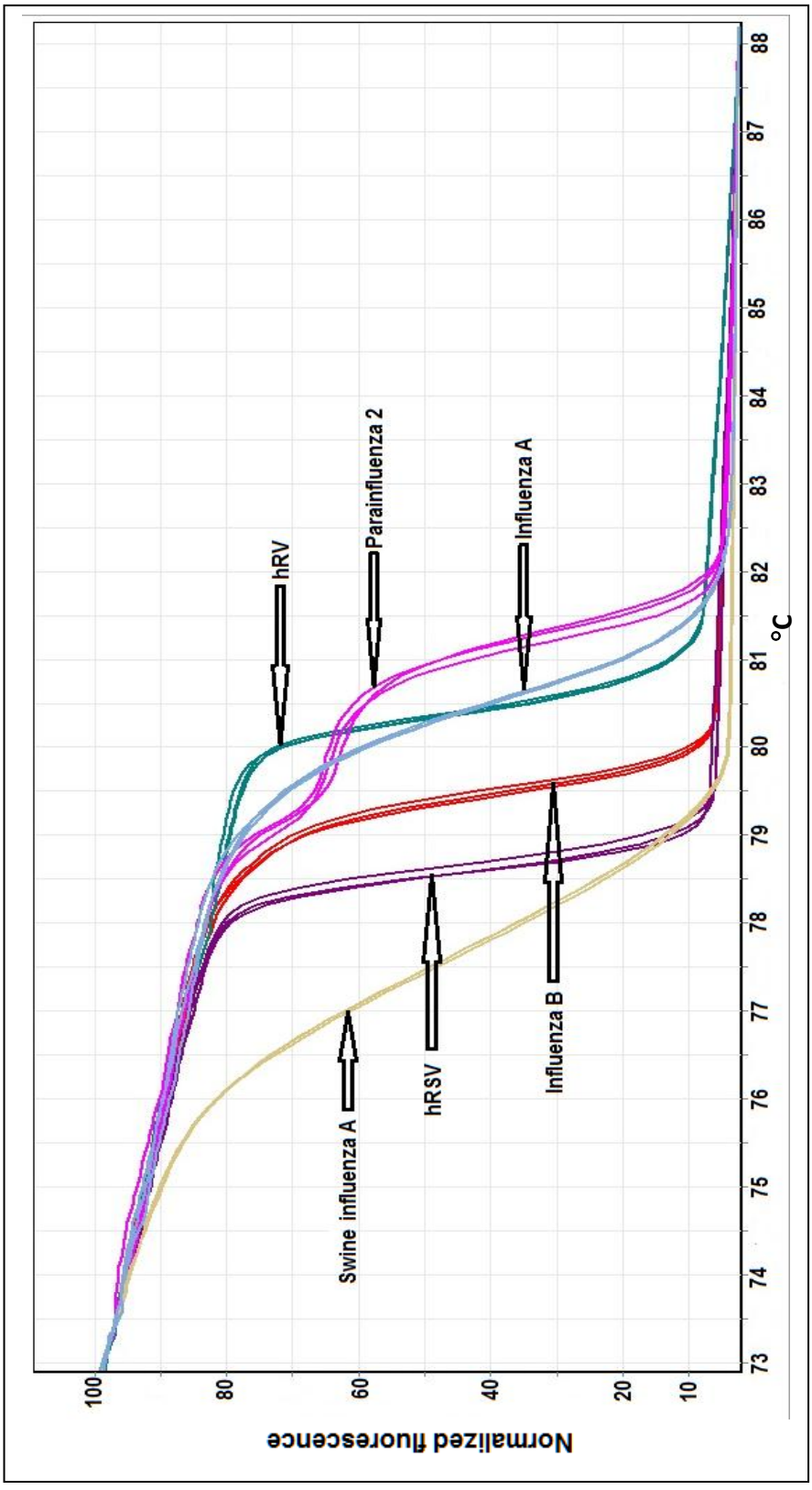

Figure 7.14: Normalized HRM analysis of the PCR amplicons from RNA virus sequences of swine influenza A, hRSV, influenza B, hRV, parainfluenza 2 and influenza A. Viruses corresponding to the curves are shown with arrows. X axis shows °C and Y axis shows normalized fluorescence. Color codes are shown in table 7.5.

After PCR and HRM analysis of different virus sequences were completed they were resolved on an agarose gel (Section 2.6.1) for confirmation of different amplicon sizes. It was found that the PCR products for influenza B, hRSV, hRV, parainfluenza 2, swine influenza A and influenza A virus sequences were 362 bp, 279 bp, 549 bp, 507 bp, 98 bp and 120 bp, respectively (Figure 7.15).

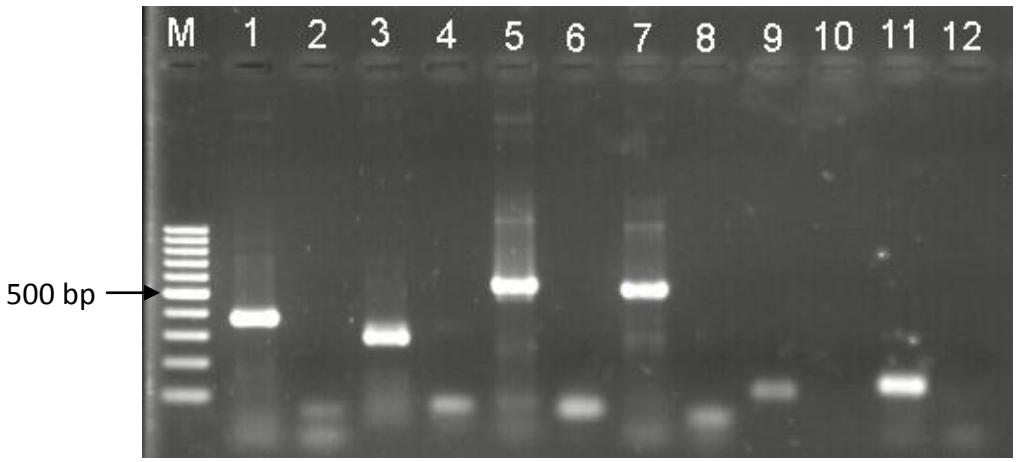

Figure 7.15: Gel electrophoresis of different PCR products of known viruses after HRM analysis. M: 100 bp DNA ladder (Fermentas, York, UK); Lane 1: influenza B (362 bp); Lane 3: sample 35 positive for hRSV (279 bp); Lane 5: sample 15 positive for hRV (549 bp); Lane 7: parainfluenza 2 (507 bp); Lane 9: swine influenza A (98 bp); Lane 11: influenza A (120 bp); Lanes 2, 4, 6, 8, 10 and 12 are negative controls for influenza B, hRSV, hRV, parainfluenza 2, swine influenza A and influenza A viruses, respectively.

7.5 Discussion

High resolution melting analysis is a very sensitive technique which can detect different types of sequences in a closed tube reaction without the need for expensive probes. It has already been reported by other workers that high resolution melting (HRM) analysis could be applied for different types of applications such as detection of bacteria (Cheng *et al.*, 2006), typing of viruses (Dames *et al.*, 2007; Sabol *et al*., 2009; Varillas *et al*., 2011) and mutational analysis (Krypuy *et al.*, 2007). This study was conducted to evaluate the applications of HRM analysis for pre-sequence screening of libraries generated after whole genome amplification from samples containing virus genomes. We also evaluated HRM for rapid detection of common pathogenic viruses of the respiratory tract.

Three separate libraries were generated from bacteriophage MS2 RNA, tissue culture media positive for influenza B and hRSV using the RT-Bst SPA as described in chapter 6 (Figure 6.1). Ten clones were picked randomly from each library for extraction of plasmids and analysis of sequences by a real-time PCR followed by HRM analysis. After matching the sequence information with the findings from the HRM analysis it was found that similar sequences produced similar melting curves and different sequences produced different melting curves from the selected clones from the 3 libraries. Although the melting curves of the different sequences of MS2 and influenza B did not produce distinct peaks in their conventional melting analyses, the normalised HRM curves produced distinct patterns for different groups of sequences after analysing the sequences with Rotor-Gene Q software 1.7 (Section 7.3.1 and 7.3.2). Normalization of the HRM curves using appropriate software for example, Rotor-Gene Q software 1.7, helps to set relative fluorescence values for all of the studied sequences uniformly from 100% to 0% after collecting them in the pre-melt and post-melt areas for all samples (Figure 7.8, 7.11 and 7.14). Normalisation of the HRM melting curves brings similar sequences closer and takes different sequences apart and helps to

recognise the similarity and differences between the sequences easily (Figure 7.8, 7.11 and 7.14).

HRM analysis and normalisation of the melting curves generated from the ten clones of MS2 and influenza B produced 3 and 4 different types of melting curves, respectively (Figure 7.8 and 7.11). MS2 sequence analysis showed that clones 1, 2 and 3; clones 6, 7, 8 and 9 and clones 4, 5 and 10 formed three separate groups. Influenza B sequence analysis showed clone 1 and 2; clones 8 and 9; clones 3, 4 and 5 and clones 6, 7 and 10 formed four distinct groups. The melting curves of hRSV was analysed using Rotor-Gene Q software 1.7 and it was found that both the melting curves and normalized HRM curves showed 5 distinct groups of sequences. Clone 1; clones 3 and 4; clones 9 and 10; clones 6, 7 and 8 and clones 2 and 3 formed 5 distinct groups (Figure 7.11). It was not possible to determine the sequence variations from conventional melting curves. However, it was possible to clearly determine the similarities and differences between different sequences using normalized HRM curves.

Generally, a large number of clones need to be sequenced randomly from a whole genome library to determine the sequence of any new type of virus (Allander *et al.*, 2005). Stang *et al*. (2005) screened the WGA library based on the different sizes of the PCR products being cloned. Application of HRM analysis for pre-sequence screening of WGA library was found to be useful in this study. This approach will enable researchers to screen different types of sequences based on the patterns of normalised HRM curves. Thereby, application of HRM for screening WGA libraries will reduce the cost for screening clones containing different types of sequences. Provaznikova *et al*., (2008) adapted HRM analysis for pre-sequence screening of myosin heavy chain (MYH) 9 gene and could reduce more than 85% of the unnecessary sequencing of MYH 9 gene.

In another part of this study cloned plasmid sequences of common pathogenic viruses of the respiratory tract, for example, hRSV, hRV, influenza B, parainfluenza 2, influenza A and swine influenza A were used to determine the

validity of HRM for rapid detection of common pathogenic viruses. After HRM analysis using Rotor-Gene Q software 1.7, it was found that each virus sequence produced distinct melting curve and HRM curve subsequent to normalization. Individual viruses also showed separate melting temperature after such analysis. Having matched the melting temperatures and normalized HRM curve of a known virus with an unknown one, it is possible to confirm the presence of a particular type of virus after HRM analysis. Fluorescence intensity generated from swine influenza A was found to be the lowest, possibly because of the shorter size (98 bp) of the amplicon and lowest melting temperature. It was also difficult to observe the small amplicon (98 bp) of swine influenza A after PCR and agarose gel electrophoresis. However, HRM analysis of the PCR product of swine influenza A virus produced a separate curve and it was not difficult to distinguish it from other viruses. A point worth mentioning in this study is that all of the above mentioned viruses were successfully amplified using the same PCR cycle, same reagent and same software. This is a new dimension of rapid and accurate detection of respiratory viruses using HRM analysis. This new approach will therefore be useful for rapid detection of any particular type of virus or a group of viruses. Appropriate positive control will be required for each type of virus to match the similarities of sequences. Any variation in the detected sequences can be further investigated by sequencing to determine any new type of the known virus. Similar results of HRM analysis have been reported by other workers for typing variants of influenza A (Lin *et al*., 2008; Varillas *et al*., 2011), human metapneumovirus (Sabol *et al*., 2009) and analysing the genetic diversity of HIV without sequencing (Towler *et al*., 2010).

A single base variation can be determined easily by HRM analysis when the amplicon size is small as was demonstrated by Liew *et al*., (2004) for SNP genotyping using a product length from 38 to 50 bp. Differences in the T_m value among the genotypes increased with the decrease in the amplicon size. It also took a short period of time for amplification of such small amplicons. Nevertheless, it was also possible to distinguish sequence variations in heterozygotes when HRM was performed for small amplicons (Graham *et al.,* 2005). HRM analysis also

carried out successfully for SNP genotyping by other scientists with relatively larger sizes of amplicons such as 160 to 218 bp (Liew *et al*., 2006 and 2007).

HRM analysis was also used for the detection and identification of 25 clinically important bacteria based on the ribosomal RNA real time PCR (Cheng *et al.*, 2006). Different sets of primers were used to differentiate between the amplicons from closely related species. HRM analysis of heteroduplex sequences is another way to differentiate between similar sequences (Sabol *et al.*, 2009). However, such an approach was not used in this study. It was possible to detect and differentiate among three species of *Aspergillus* sequences having very similar T_ms by introducing tailed primers in a multiplex assay (Erali *et al.*, 2006). HRM was also applied for analysing pathogenic microorganisms such as genotyping *Staphylococcus aureus* (Stephens *et al.*, 2008), typing variants of influenza A (Lin *et al*., 2008; Varillas *et al*., 2011) and detecting strains of *Bacillus anthracis* (Fortini *et al*., 2007). In recent studies it was also possible to determine oseltamivir drug-resistance using HRM analysis for some isolates of the influenza A/H1N1 2009 viruses (Lee *et al*., 2011; Tong *et al*., 2011).

HRM analysis of amplicons was also found to be useful for determining mutation in the DNA sequences. It was possible to detect 91% of gastrointestinal stroma tumours with abnormal melting curves after HRM analysis (Holden *et al*., 2007). Other workers also reported the application of HRM analysis for determining the DNA methylation patterns (Dahl and Guldberg, 2007). However, data analysis of curves generated after HRM analysis is still criticized due to the lack of statistical interrogation and arbitrary interpretation. Recently, a software program called ‘ScreenCLust’ has been developed for improved statistical analysis of HRM data (Reja *et al*., 2010). Samples can be analysed based on the likelihood of the sequences in both supervised and unsupervised modes in the presence of known and unknown controls, respectively. It is possible to analyse data without known controls using this new algorithms hence it has the potential for *de novo* HRM applications such as mutation discovery. This new software could be further

applied for detailed analysis of insertions, deletions, pathogen detection and methylation analysis (Reja *et al*., 2010).

The HRM analysis was suitable for determining variations in the nucleotide sequences in a library generated after WGA in this study. It was also possible to determine variations in the nucleotide sequences of known viruses. Although the amplicon sizes varied between 90 bp and 507 bp for PCR amplification and HRM analysis of pathogenic viruses, it was possible to differentiate the sequences of known viruses using this technique. HRM is a closed tube reaction which reduces the possibility of carry over contamination and unnecessary expenses for sequencing the amplified products. A panel of known viruses can be used to further optimize this technique for rapid screening of a panel of common pathogenic viruses of the respiratory tract. Further optimisation of HRM analysis will aid rapid detection of pathogenic viruses of the respiratory tract leading to control of the spread and intervention of infectious diseases, as well as cost-effective management of patients.

Chapter 8

Discussion and future work

8.1 Introduction

This study evaluates the application of isothermal amplification of nucleic acids for the detection and characterization of pathogenic viruses of the respiratory tract. In the preliminary study on phi29-amplification of four different structural conformations of DNA it was found that linear and circular forms of double stranded DNA were amplified whereas single stranded DNA (ssDNA) was not amplified. This was reflected when single stranded DNA was synthesized from single stranded bacteriophage MS2 RNA chosen as the representative genome of single stranded RNA viruses. Since recognised pathogenic viruses of the respiratory tract are generally RNA viruses, with the exception of adenoviruses, the inability of phi29 to amplify ssDNA was a disadvantage. This indicates that alternative protocols are necessary for amplification of sscDNA reverse transcribed from single stranded viral RNA. Therefore, we evaluated alternative protocols for isothermal amplification of single stranded cDNA (prepared from a RNA template) to enhance the sensitivity of detection by PCR. Ligation-mediated phi29-amplification was found to be helpful for the amplification of ssDNA but was dependent on the reverse transcription of cDNA from viral RNA. A novel protocol designated RT-Bst was developed for the simultaneous reverse transcription of RNA and amplification of cDNA to enhance the sensitivity of PCR detection of pathogenic viruses of the respiratory tract. This RT-Bst amplification was applied for the amplification of RNA extracted from bacteriophage MS2 and tissue culture media extracts positive for hRSV which showed significant amplification in qualitative PCR assays (discussed in Chapter 4). The performance of RT-Bst amplification varied when it was used for the amplification of RNA extracted from nasopharyngeal samples (discussed in Chapter 5). An alternative and shorter protocol for amplification of viral RNA was also developed in this study and named RT-Bst SPA to aid nucleic acid based detection of pathogenic viruses of the respiratory tract (Chapter 6; Table 6.5). RT-Bst SPA amplified products were subcloned to make whole genome libraries. The cloned sequences were analysed using the BLASTn tool to determine the utility of RT-Bst SPA for whole genome amplification. In sequence based analysis, a large number of clones are randomly selected to determine the whole genome sequences which is tedious,

lengthy and expensive. Therefore, an alternative approach of High Resolution Melting (HRM) analysis was used to evaluate its application for pre-sequence screening of a whole genome library to avoid unnecessary sequencing. PCR based detection of pathogenic microorganisms requires sequencing or use of nucleic acid probes for confirmation of the amplified nucleic acid sequences which can be lengthy and/or expensive. In this study we demonstrated the applications of HRM analysis as an alternative approach to sequencing for detection and characterization of common pathogenic viruses of the respiratory tract.

8.2 Approaches for cell-free isothermal amplification of nucleic acids and their applications for enrichment of viral genomes

Different approaches of isothermal amplification of nucleic acids were evaluated in this study to aid PCR detection of pathogenic viruses of the respiratory tract (Chapter 4 and 6). Isothermal amplification of nucleic acids was used in this study because it is less biased over the PCR based amplification of the template DNA. These approaches can also be used for amplification of nucleic acids from environmental, biological and clinical samples. As the aim of our study was to amplify viral RNA, we developed a modified protocol (ligation-mediated phi29-amplification) for amplification of single stranded DNA (prepared from RNA). We used T4 RNA ligase, T4 DNA ligase and CircLigase II ssDNA ligase for ligation mediated phi29-amplification using MS2 RNA as substrate. Based on our findings, ligation-mediate phi29-amplification was found to be useful for the amplification of ssDNA. Further studies will be required for the application of the ligation-mediated phi29-amplification protocol for detection of viral RNA in clinical, biological and environmental samples.

Phi29 DNA polymerase amplifies low copy number templates to elevate the concentration of DNA above the normal detection limit of PCR. This is expected to enhance the sensitivity of PCR detection and reduce the number of false negative results. Sequence independent isothermal amplification of nucleic

acids in clinical samples using similar approaches is expected to improve sensitivity of molecular detection of pathogenic viruses of the respiratory tract. As in the present study, phi29-amplifiction has also been found to be useful by other workers for amplification of DNA and RNA viruses in clinical samples for subsequent microarray analysis (Erlandsson *et al*., 2011 and 2010). Erlandsson *et al*., (2011) used phi29 amplification for amplification of DNA (for example, herpes simplex virus) and RNA viruses (for example, rotavirus, hepatitis C virus, hRSV and enterovirus) from a diverse set of clinical samples such as, cerebrospinal fluid, urine, serum, faeces, lesion and tracheal aspirate for successful amplification and identification by microarray analysis.

In this study we developed a novel method named RT-Bst for the simultaneous reverse transcription of RNA and *Bst* DNA polymerase amplification of cDNA in the same reaction (Section 4.4). In this study, amplification of cDNA copy using RT-Bst reaction was found to be useful to improve the sensitivity of PCR detection of RNA viruses. In a qualitative PCR based assay on bacteriophage MS2 RNA it was found that cDNA molecules at nanogram levels could be amplified more efficiently using the RT-Bst protocol than the QuantiTect™ reverse transcription kit (QIAGEN, Crawley, UK) (Section 4.4). A more detailed quantitative real-time PCR assay will be required for accurate determination of RT-Bst amplification from limiting concentrations of RNA alone and in the presence of carrier RNA to determine the sensitivity of the RT-Bst amplification. The RevertAid™ Premium (thermostable form) reverse transcriptase (Fermentas, York, UK) was used for RT-Bst amplification of cDNA from RNA extracted from nasopharyngeal samples (Chapter 5). In this study the performance of RT-Bst PCR was found to be comparable to that of one-step RT-PCR detection and more efficient than the immunofluorescence detection of common pathogenic viruses of the respiratory tract by the Chelsea and Westminster Hospital (Tables 5.1, 5.2, 5.3 and 5.4). The performance of RT-Bst amplification could be further improved by optimising the reaction temperature (50-60 ºC) and designing appropriate primers (preferably in the central portion of the genome) for detection of virus sequences by PCR. In this study we determined the RT-Bst amplification of hRSV genomic RNA using eight pairs of primers in a qualitative PCR and we found that the central portion of the hRSV

genome was amplified more efficiently than the terminal parts (Section 4.4.2). In future studies RT-Bst amplification could also be improved through modification of the reaction conditions for example, allowing only reverse transcription in the first 15-30 minutes at 50-60 ºC using genetically modified RNase H deficient thermostable RevertAid™ premium reverse transcriptase (Fermentas, York, UK). Subsequently RNase H and *Bst* DNA polymerase could be added for simultaneous degradation of RNA from cDNA:RNA hybrid and amplification of cDNA at 60-65 ºC for 1-2 hours. These controlled reaction conditions will increase the stability of RNA in the first 30 minutes for efficient reverse transcription and degradation of RNA afterwards from the cDNA:RNA hybrid for efficient *Bst* DNA polymerase amplification of cDNA. If such a modified protocol is adopted, it could be used for the development of an alternative reverse transcription kit to those available in the market. In commercially available reverse transcription kits only a fraction of RNA (40-80%) is converted to cDNA whereas the RT-Bst reaction not only converts cDNA from RNA but also amplifies the concentration of cDNA in the reaction (Stangegaard *et al*., 2006). RT-Bst amplification of RNA from minute amounts will enable scientists to further study any type of RNA sample such as from virus, bacteria or even eukaryotic RNA. This approach can be applicable for amplification of nucleic acids from both the culturable and unculturable forms of bacteria using molecular detection techniques for example, PCR, hybridization, microarray analysis. RT-Bst and RT-Bst SPA amplification can be useful for amplification of nucleic acids from laser captured microdissected tissues (Aaltonen *et al*., 2011).

A significantly large amount of DNA could be prepared using RT-Bst and RT-Bst SPA from a small amount of clinical sample for detection of pathogenic viruses using the multiplex detection techniques for example, ResPlex™ technology (Brunstein *et al*., 2008) and Infinity™ system (Raymond *et al*., 2009). Recently, Erlandsson *et al*., (2010) used a similar strategy for pre-amplification of circular and linear dsDNA genomes of viruses using phi29 DNA polymerase to increase the sensitivity of real-time PCR detection. Erlandsson *et al*., (2011) also demonstrated phi29-amplification for enrichment of RNA and DNA viruses for subsequent detection by microarray analysis. In our study we

demonstrated a unique application of the RT-Bst method for the amplification of nucleic acids directly from RNA in a single tube reaction (Erlandsson *et al*., 2011 and 2010) (Chapter 4). Erlandsson *et al*., (2011, 2010) used the Whole Transcriptome Amplification (WTA) kit (QIAGEN, Crawley, UK) for phi29-amplification of viral RNA which may not be useful for diagnostic laboratories in terms of cost and time. The WTA kit requires multiple steps for ligation of RNA and amplification of cDNA which is prone to contamination from any template DNA from the laboratory. On the contrary, the RT-Bst reaction is a closed tube reaction and, as a result, is less prone to contamination from external sources. In routine laboratory diagnosis, nested-PCR (Perrott *et al*., 2009) may be used to increase the sensitivity of PCR detection of samples containing very low copies of the template (Schloss *et al*., 2003). However, nested-PCR amplification represents high risk of amplicon contamination leading to potentially false positive results (Templeton *et al*., 2004; Bellau-Pujol *et al*., 2005).

8.3 RT-Bst SPA—a novel approach for sequence independent nucleic acid amplification

A sequence independent approach can be used for the amplification of both known and unknown virus sequences. Although PCR-based methods are considered to be more biased than non-PCR based isothermal amplification methods, the former is more suitable for cloning and preparation of libraries than the latter. Isothermal amplification of nucleic acids produces hyper-branched structures of DNA which require additional steps for converting them to dsDNA before cloning to make a genomic library (Zhang *et al*., 2006). In this study we designed a novel method by combining RT-Bst amplification of cDNA from RNA samples followed by Single Primer Amplification (RT-Bst SPA) (Chapter 6, Figure 6.1). The RT-Bst SPA designed in this study involves fewer steps and is less costly compared to the SISPA technique for amplification of whole genomes of RNA viruses which has been adopted in many studies (Table 6.5). Our method for the amplification of whole genome requires a relatively lower volume of sample (50 µl) compared to other methods, for

example, SISPA (200 µl) (Allander *et al*., 2001 and 2005) and particle associated nucleic acid PCR (10 ml) (Stang *et al*., 2005) (Section 6.5). However, for virus amplification a pre-treatment with DNase was found to be critical for the appropriate digestion and removal of host DNA and other unwanted sequences. Further studies will be required to optimise DNase treatment for digestion of cell-free DNA before whole genome amplification. Allander *et al*., (2001) optimised DNase SISPA technique on hepatitis B virus, bacteriophage M13 and GBV-B viruses for amplification of whole genome sequences. They found a smear of different sizes of PCR products after SISPA without any DNase treatment which was resolved to discrete PCR bands after optimum DNase treatment of the samples. A similar approach of DNase treatment can be optimised for whole genome amplification from nasopharyngeal samples as a future direction of this study.

In the present study (Chapter 4) we used the RT-Bst amplified products as templates for subsequent PCR amplification. It was found that the RT-Bst amplified products can be used directly at 10% of the PCR reaction volume for amplification of cDNA sequences (Chapter 4 and 5). Addition of RT-Bst amplified product at 10% of a real-time PCR reaction showed inhibition in PCR amplification. A further dilution (1:10) of the RT-Bst amplified products before PCR amplification showed no inhibition in real-time PCR amplification (data not shown). However, further studies will be required to determine the optimum dilution for real-time PCR amplification of RT-Bst amplified products.

RT-Bst SPA has been used in this study for successful amplification of genomic RNA of bacteriophage MS2. Both the strategies of whole genome amplification (RT-Bst SPA) using random and sequence specific primers (including tail sequences) could be useful for microarray analysis and multiplex PCR based detection technology for example, ResPlex™ and Infinity™ system for detection of pathogenic viruses of the respiratory tract. Most of the microarray analysis is not sensitive enough for detection of pathogenic microorganisms present in low concentration and usually requires pre-amplification of nucleic acids such as, using PCR or virus culture. Both RT-Bst and RT-Bst SPA amplified nucleic acids can also be used for Next Generation Sequencing (Section 1.8.4) and detection of previously uncharacterized virus sequences. RT-Bst SPA can also

be used for pre-enrichment of genomic sequences for efficient multiplex detection of pathogenic viruses using conventional PCR and real-time PCR.

8.4 Application of HRM analysis for rapid detection of virus sequences

In this study (Chapter 7) we investigated the applications of HRM analysis for screening sequences from whole genome libraries and rapid detection of common pathogenic viruses of the respiratory tract. After PCR amplification of the inserts in randomly selected clones and HRM analysis it was found that the normalised HRM curves showed reproducible and predictable shapes for related sequences allowing the differentiation of sequences into different groups based on their similarity (Figures 7.4, 7.6 and 7.8). In the present investigation 30 cloned sequences were screened from MS2, influenza B and hRSV libraries using HRM analysis. These 30 clone sequences produced 12 different types of melting curves which means that there were 12 types of sequences within the 30 clones. Based on the results of this study, it may be possible to save 60% of the unnecessary sequencing of these 30 clones from three libraries in this study if they were screened by HRM analysis. Other workers also reported the possibility of avoiding unnecessary sequencing of more than 85% of the MHY9 gene using HRM analysis for pre-sequence screening (Provaznikova *et al*., 2008). The combined application of RT-Bst SPA for whole genome amplification and HRM analysis for pre-sequencing will be a potentially useful combination for sequence based analysis and detection of new types of viruses.

HRM analysis has been used in previous studies for detection of bacteria (Cheng *et al.*, 2006), typing viruses (Dames *et al.*, 2007; Sabol *et al*., 2009; Varillas *et al*., 2011) and mutational analysis (Krypuy *et al.*, 2006). In this study (Chapter 7) we used HRM analysis for rapid and accurate detection of common pathogenic viruses of the respiratory tract. It was found that influenza A, swine influenza A, influenza B, hRSV, parainfluenza 2 and hRV produced distinct curves after HRM analysis. It was also possible to run all samples using the

same cycling program. Further optimisation of HRM analysis would be useful for detection of a panel of viruses from the same sample using the same cycling conditions. Including positive controls for virus types of interest can help to interpret the HRM curves generated from unknown samples. Additionally, template concentrations below the optimum concentration for PCR amplification followed by the HRM analysis (C_T value less than 30) can be enriched by our developed methods, RT-Bst (Chapter 4) or RT-Bst SPA (Chapter 6). In this study purified plasmid constructs of known concentrations were used for PCR amplification followed by HRM analysis. In the future, a more detailed study will be required for validation of HRM analysis on nasopharyngeal samples for the detection and characterization of a panel of pathogenic viruses of the respiratory tract. RT-Bst and RT-Bst SPA could be used for enrichment of low copy number of viral sequences from clinical and environmental samples. It may be suggested that HRM analysis in combination with our developed methods RT-Bst and RT-Bst SPA could be a potentially rapid and sensitive approach for the detection of a panel of pathogenic viruses of the respiratory tract.

8.5 Conclusion

This study was designed to evaluate the applications of isothermal nucleic acid amplification methods using phi29 and *Bst* DNA polymerases to enrich template DNA and RNA from low copy to enhance the detection level of different types of nucleic acids. Phi29 amplification alone was found to be useful in this study and by other workers (Hosono *et al.*, 2003; Lasken *et al.*, 2004) for amplification of double stranded DNA. Alternative protocols of ligation-mediated phi29-amplification were found to be suitable for amplification of single stranded DNA. Two novel protocols, RT-Bst and RT-Bst SPA were developed during this study for sequence independent amplification of viral nucleic acid sequences. The RT-Bst and RT-Bst SPA methods can be used for isothermal amplification of cDNA from RNA genomes to increase the sensitivity of PCR, microarray and hybridisation based detection and characterization of pathogenic viruses (Figure 8.1). Amplified whole genome using RT-Bst SPA

can be used for Next Generation Sequencing (Section 1.8.4) for the detection of currently undescribed viruses. A summary of the different approaches and their applications are shown in figure 8.1. Although the RT-Bst and RT-Bst SPA approaches were used on RNA virus genomes in this study, these could be adapted for detection and categorization of different types of viral nucleic acids for example, circular DNA and double stranded RNA. Development of a miniature version of these isothermal amplification approaches will be a step forward for on-site rapid detection of viral nucleic acids. Highly specific amplification is crucial for accurate detection from limiting number of nucleic acid molecules. Most of the existing miniaturised nucleic acid analysis systems use PCR amplification. The application and potential of miniaturised isothermal nucleic acid amplification methods for example, NASBA, LAMP, HAD, RCA and SDA have recently been demonstrated by other workers (reviewed by Asiello and Baeumner, 2011). The next step in the development of miniaturised systems may be the development of ready-to-use disposable µTAS (micro total analysis system) where reagents and primers can be pre-stored on the chip (reviewed by Asiello and Baeumner, 2011).

A quantitative real-time PCR assay can be performed to determine the magnitude of isothermal amplification of nucleic acid sequences using these novel methods. RT-Bst and RT-Bst SPA protocols can be further improved through optimisation of the reaction conditions and modification of enzymes. A more elaborate study including a larger number of clinical samples will be required to validate the applications of RT-Bst and RT-Bst SPA for multiplex detection of pathogenic viruses of the respiratory tract. Further optimisation of DNase treatment will be required for enhanced whole genome amplification using RT-Bst SPA for the detection and characterization of undescribed viruses. The novel methods RT-Bst and RT-Bst SPA developed in this study are competitive to other kits in the market used for reverse transcription and whole genome amplification in terms of their performances. However, further validation of the protocols on a larger number of clinical samples will be required for appropriate commercialisation of the approaches developed in this study.

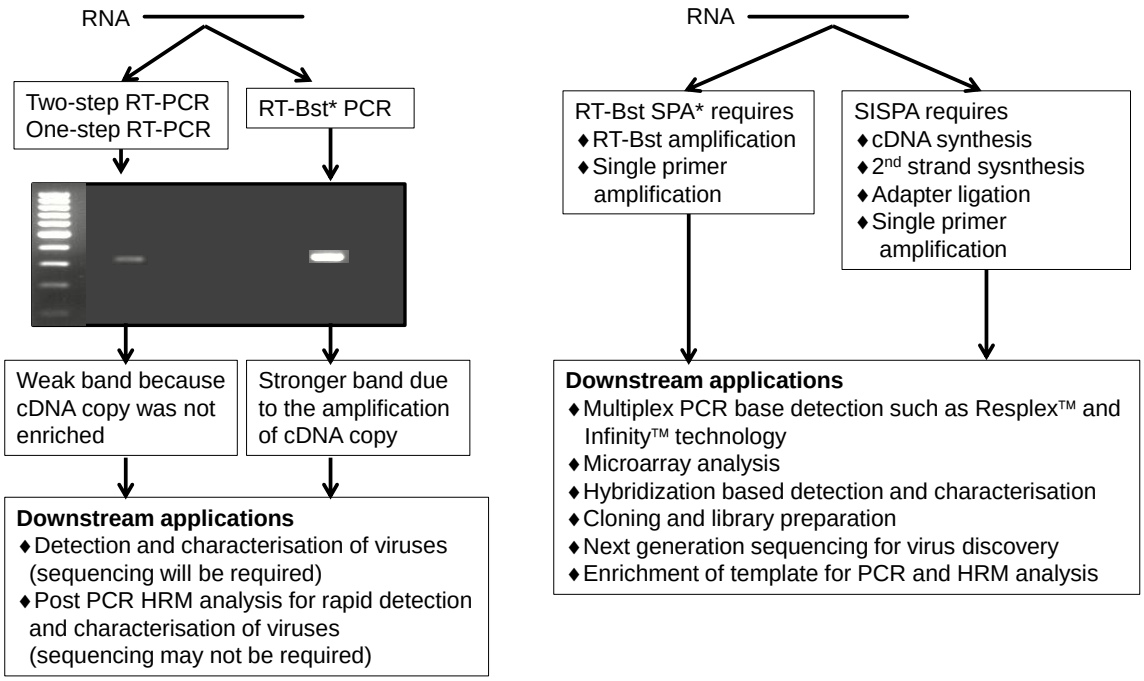

Figure 8.1: Flow chart for the application of existing and novel protocols (*RT-Bst and *RT-Bst SPA) developed in this study for detection and characterization of pathogenic viruses

Chapter 9

References

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Appendices

Appendix i

Table A1: Primer sequences used for detection influenza A virus detection

Primer name	Primer sequence (5'-3')	Source
SW InfA Forward	5'-GCACGGTCAGCACTTATYCTRAG-3'	WHO, IDT
SW InfA Reverse	5'-GTGRGCTGGGTTTTCATTTGGTC-3'	WHO, IDT
SW H1 Forward	5'-GTGCTATAAACACCAGCCTYCCA-3'	WHO, IDT
SW H1 Reverse	5'-CGGGATATTCCTTAATCCTGTRGC-3'	WHO, IDT
InfA Forward	5'-GAC CRA TCC TGTCACCTCTGAC-3'	WHO, IDT
InfA Reverse	5'-AGGGCATTYTGGACAAAKCGTCTA	WHO, IDT
Randdom hexamer	5'-NNNNNN-3'	IDT
Pentadecamer random primer	5'-NNNNNNNNNNNNN-3'	IDT
Lambda-F	5'-GATGAGTTCGTGTCCGTACAACTGG-3'	IDT
Lambda-R	5'-GGTTATCGAAATCAGCCACAGCGCC-3'	IDT

Appendix ii

Whole genome library sequences:

Whole genome library prepared from MS2 RNA (tail primer in bold and italic)

>MS2-Clone-1-MS2sequence-2592-2911-320bp

GACCATCTAGCGACCTCCACATAAAAGTGCGCGCCGCAGCTCTCGCGAAAGA
GCCCGGACACGAACGTTTTACGAAGATTCGGTTTAAAACCGTAGTAGGCAAGT
GCCTCTAGCACACGGGGTGCAATCTCACTGGGACATATAATATCGTCCCCGTA
GATGCCTATGGTTCCGGCGTTACCAAAATGGATTTGGGTCGCTTTGACTATTGC
CCAGAATATCATGGACTCTAGCTCAAATGTGAACCCATTTCCCATTGTGGAAAAT
AGTTCCCATCGTATCGTCTCGCCATCTACGATTCCGTAGTGTGAGCGGATACGA
TCGAGATATGAATATAGCTcg***GTGGAGGTCGCTAGATGGTC***

>MS2-Clone-2-MS2sequence-2592-2911-320bp

GACCATCTAGCGACCTCCACATAAAAGTGCGCGCCGCAGCTCTCGCGAAAGA
GCCCGGACACGAACGTTTTACGAAGATTCGGTTTAAAACCGTAGTAGGCAAGT
GCCTCTAGCACACGGGGTGCAATCTCACTGGGACATATAATATCGTCCCCGTA
GATGCCTATGGTTCCGGCGTTACCAAAATGGATTTGGGTCGCTTTGACTATTGC
CCAGAATATCATGGACTCTAGCTCAAATGTGAACCCATTTCCCATTGTGGAAAAT
AGTTCCCATCGTATCGTCTCGCCATCTACGATTCCGTAGTGTGAGCGGATACGA
TCGAGATATGAATATAGCTccG***GTGGAGGTCGCTAGATGGTC***

>MS2-Clone-3-MS2sequence-2590-2911-322bp

GACCATCTAGCGACCTCCACATAAAAGTGCGCGCCGCAGCTCTCGCGAAAGA
GCCCGGACACGAACGTTTTACGAAGATTCGGTTTAAAACCGTAGTAGGCAAGT
GCCTCTAGCACACGGGGTGCAATCTCACTGGGACATATAATATCGTCCCCGTA
GATGCCTATGGTTCCGGCGTTACCAAAATGGATTTGGGTCGCTTTGACTATTGC
CCAGAATATCATGGACTCTAGCTCAAATGTGAACCCATTTCCCATTGTGGAAAAT
AGTTCCCATCGTATCGTCTCGCCATCTACGATTCCGTAGTGTGAGCGGATACGA
TCGAGATATGAATATAGCTcag***GTGGAGGTCGCTAGATGGTC***

>MS2-Clone-4-MS2sequence-2590-2918-329bp

GACCATCTAGCGACCTCCACCTGAGCTATATTCATATCTCGATCGTATCCGCTC
ACACTACGGAATCGTAGATGGCGAGACGATACGATGGGAACTATTTTCCACAAT
GGGAAATGGGTTCACATTTGAGCTAGAGTCCATGATATTCTGGGCAATAGTCAA
AGCGACCCAAATCCATTTTGGTAACGCCGGAACCATAGGCATCTACGGGGACG
ATATTATATGTCCCAGTGAGATTGCACCTCGTGTGCTAGAGGCACTTGCCTACT
ACGGTTTTAAACCGAATCTTCGTAAAACGTTCGTGTCCGGGCTCTTTCGCGAGA
GCTGCGGCGCGCACTTTTACTGTGGT***GTGGAGGTCGCTAGATGGTC***

>MS2-Clone-5-MS2sequence-2590-2918-329bp

GACCATCTAGCGACCTCCACCTGAGCTATATTCATATCTCGATCGTATCCGCTC
ACACTACGGAATCGTAGATGGCGAGACGATACGATGGGAACTATTTTCCACAAT
GGGAAATGGGTTCACATTTGAGCTAGAGTCCATGATATTCTGGGCAATAGTCAA
AGCGACCCAAATCCATTTTGGTAACGCCGGAACCATAGGCATCTACGGGGACG
ATATTATATGTCCCAGTGAGATTGCACCTCGTGTGCTAGAGGCACTTGCCTACT
ACGGTTTTAAACCGAATCTTCGTAAAACGTTCGTGTCCGGGCTCTTTCGCGAGA
GCTGCGGCGCGCACTTTTACTGTGGT***GTGGAGGTCGCTAGATGGTC***

>MS2-Clone-6-MS2sequences-1154-1370-217bp

GACCATCTAGCGACCTCCACCTACGCGAaGTTGCTTGGGgCGACAGtCACGtcga
gTTCCGCCATTGTCGACGAGAACGAACTGAGTAGAGTTAGAAGCCATGcTTCAA
ACTCCGGTTGAGGGCTCTATCTAGAGAGCCGTTGCCTGATTAATGCTAACGCAT
CTAAGGTATGGACCATCGAGAAAGGGGaCTTTACGTACGCGCCAGTTGTTGGC
CATACGGATTgTACCCCTCGATG***GTGGAGGTCGCTAGATGg***

>MS2-Clone-7-MS2sequences-1154-1353-200bp

TACGCGAAGTTGCTTGGGGCGACAGTCACGTCGCCAGTTCCGCCATTGTCGAC
GAGAACGAACTGAGTAGAGTTAGAAGCCATGCTTCAAACTCCGGTTGAGGGCT
CTATCTAGAGAGCCGTTGCCTGATTAATGCTAACGCATCTAAGGTATGGACCAT
CGAGAAAGGGGACTTTACGTACGCGCCAGTTGTTGGCCATACGGATTGTACCC
CTCGATGGTGGAGGTCGCTAGATGGTC***AAGGGCGAATTCCAGCACAC***

>MS2-Clone-8-MS2sequences-1154-1370-217bp

GACCATCTAGCGACCTCCACCTACGCGAAGtTGCTTGGGgcGACAGTCACGtcga
gTTCCGCCATTGTCGACGAGAACGAACTGAGTAGAGTTAGAAGCCATgcTTCAAA
CTCCGGTTGAGGGCTCTATCTAGAGAGCCGTTgcCTGATTAATGCTAACGCATC
TAAGGTATGGACCATCGAGAAAGGGGAcTTTACGTACGCGCCAGTTGTTGGcCA
TACGGATTGTACCCCTCGATG***GTGGAGGtCgcTAGATGgtC***

>MS2-Clone-9-MS2sequences-1154-1370-217bp

GACCATCTAGCGACCTCCACCTACGCGAAGTTGCTTGGGGCGACAGTCACGT
CGCCAGTTCCGCCATTGTCGACGAGAACGAACTGAGTAGAGTTAGAAGCCATG
CTTCAAACTCCGGTTGAGGGCTCTATCTAGAGAGCCGTTGCCTGATTAATGCTA
ACGCATCTAAGGTATGGACCATCGAGAAAGGGGACTTTACGTACGCGCCAGTT
GTTGGCCATACGGATTGTACCCCTCGATG***GTGGAGGTCGCTAGATGGTC***

>MS2-Clone-10-MS2sequences-2590-2918-329bp

GACCATCTAGCGACCTCCACCTGAGCTATATTCATATCTCGATCGTATCCGCTC
ACACTACGGAATCGTAGATGGCGAGACGATACGATGGGAACTATTTTCCACAAT
GGGAAATGGGTTCACATTTGAGCTAGAGTCCATGATATTCTGGGCAATAGTCAA
AGCGACCCAAATCCATTTTGGTAACGCCGGAACCATAGGCATCTACGGGGACG

ATATTATATGTCCCAGTGAGATTGCACCTCGTGTGCTAGAGGCACTTGCCTACT
ACGGTTTTAAACCGAATCTTCGTAAAACGTTCGTGTCCGGGCTCTTTCGCGAGA
GCTGCGGCGCGCACTTTTACTGTGGT***GTGGAGGTCGCTAGATGGTC***

Appendix iii

Whole genome library prepared from influenza B RNA (tail primer in bold and italic)

>Influenza-B-Clone-1-**InfB-Seg1-21-23bp**-Uncultured-Eukaryotic-28S-rRNA

GaCCaTCTAGCGaCCTCCACAGCCCAGGGAGCggCTTGGaATAATCAGCGgGGA
AAGAAGACCCTGTTGAGCTTGaCTCTAGTCCGACTTGAAATGACTTTCGAGATA
TAGCCTAAGTGGGAGCTAACGCACCGTGtAaAACCACTGCTCGCTTAGCCATTT
TACTTACTCCGTGaAGAAGTGGCCAATTTATTGGTTCTTAGATTTAAACATCTATT
TTATAGGtGtGaACTTTGCGGaGgaCaAGGtGaG***GtGgAGgtCGCTAGaTG***

>Influenza-B-Clone-2-**InfB-Seg1-21-23bp**-Uncultured-Eukaryotic-28S-rRNA

GACCATCTAGCGACCTCCACAGCCCAGGGAGCGGGCTTGGAATAATCAGCGG
GGAAAGAAGACCCTGTTGAGCTTGACTCTAGTCCGACTTGAAATGACTTTCGAG
ATATAGCCTAAGTGGGAGCTAACGCACCGCTGTAAAACCACTGCTCGCTTAGC
CATTTTACTTACTCCGTGAAGAAGTGGCCAATTTATTGGTTCTTAGATTTAAACA
TCTATTTTATAGGTGTGAACTTTGCGGAGGACAAGGTGAG***GTGGAGGTCGCTA
GATGGTC***

>Influenza-B-Clone-3-Partially-Eukaryotic-**InfB-Seg-8-24-27bp**

gaCCATCTAGCGACCTCCACCCGCAACTTCTCCcATTGCTTCAAAAACTGGAAA
GTTGCTACCTGCGTTGGTATACATACTACCAACTGTTACCAAAACTGCATAAGC
ATACCCTTGGTCAACGGATTGATACATTGTTTATAGAAAGCATTGAGGCTATTTC
CATCGCAAGCTTTCTCTCTCGCGAGGAAAAGCATCCACATGTCCGCTTGGCAAT
ACGAAAAGTGGATACCTTGCGTATACTCCTGATGGTGCT***GTGGAGGTCGCTAG
ATGGTC***

>Influenza-B-Clone-4-**InfB-Seg-8-24-27bp**-Partially-Eukaryotic

GACCATCTAGCGACCTCCACCCGCAACTTCTCCCATTGCTTCAAAAACTGGAAA
GTTgCTACCTGCGTTGGTATACATACTACCAACTGTTACCAAAACTGCATAAGCA
TACCCTTGGTCAACGGATTGATACATTGTTTATAGAAAGCATTGAGGCTATTTCC
ATCGCAAGCTTTCTCTCTCGCGAGGAAAAGCATCCACATGTCCGCTTGGCAATA
CGAAAAGTGGATACCTTGCGTATACTCCTGATGGTGCT***GTGGAGGTCGCTAGA
TGGTC***

>Influenza-B-Clone-5-NoMatch-**InfB-Seg-8-24-27bp**

GACCATCTAGCGACCTCCACCCGCAACTTCTCCCATTGCTTCAAAAACTGGAAA
GTTGCTACCTGCGTTGGTATACATACTACCAACTGTTACCAAAACTGCATAAGC
ATACCCTTGGTCAACGGATTGATACATTGTTTATAGAAAGCATTGAGGCTATTTC
CATCGCAAGCTTTCTCTCTCGCGAGGAAAAGCATCCACATGTCCGCTTGGCAAT
ACGAAAAGTGGATACCTTGCGTATACTCCTGATGGTGCT***GTGGAGGTCGCTAG
ATGGTC***

>Influenza-B-Clone-6-Partly-Eukaryotic-Otherwise-**NoMatch**

GACCATCTAGCGACCTCCACCCGCTCTGATTAATCCaGTTTTCACACCCAATGT
CGGTCGATACGATCCAGTTACGCACATCTGGAGCGGACTCAATCTGGCAAGTG
AGCAAAGCATTTTCATGACGCTCACAGGCACAATCAACTCAACGACGACCGGTA
TTTTGCTGAACACGGTCAGCGTAAGGCCACCTGCGGACACCACCGACCCGAAC
GCATTGGATAACAGCGCTACCGACGCGACCTTGATCGAAGCTCGGGCCT***GTGG***
AGGTCGCTAGATGGTC

>Influenza-B-Clone-7-PartlyEukaryotic-Otherwise-**NoMatch**

GACCATCTAGCGACCTCCACCCGCTCTGATTAATCCAGTTTTCACACCCAATGT
CGGTCGATACGATCCAGTTACGCACATCTGGAGCGGACTCAATCTGGCAAGTG
AGCAAAGCATTTTCATGACGCTCACAGGCACAATCAACTCAACGACGACCGGTA
TTTTGCTGAACACGGTCAGCGTAAGGCCACCTGCGGACACCACCGACCCGAAC
GCATTGGATAACAGCGCTACCGACGCGACCTTGATCGAAGCTCGGGCCT***GTGG***
AGGTCGCTAGATGGTC

>Influenza-B-Clone-8-PartlyEukaryotic

GACCATCTAGCGACCTCCACCGTACAGAACCACCATTAGCACCATTACCGCCA
GCATAATAAGTACCATAGCCGCCGCCCGTTGCAGTAATTGTATCAAAAACAGAA
TTAGATCCCGATACCCCATTGTTGGTACCAGCCGCAGCACCGTTTCCACCCGC
ACCAATAGTCACAGTAAATGATCCGGTTGAATAAGTAGCTCCTGGGGTT***GTGGA***
GGTCGCTAGATGGTC

>Influenza-B-Clone-9-Partly-Eukaryotic

GACCATCTAGCGACCTCCACCGTACAGAACCACCATTAGCACCATTACCGCCA
GCATAATAAGTACCATAGCCGCCGCCCGTTGCAGTAATTGTATCAAAAACAGAA
TTAGATCCCGATACCCCATTGTTGGTACCAGCCGCAGCACCGTTTCCACCCGC
ACCAATAGTCACAGTAAATGATCCGGTTGAATAAGTAGCTCCTGGGGTT***GTGGA***
GGTCGCTAGATGGTC

>Influenza-B-Clone-10-NoMatch

GACCATCTAGCGACCTCCACCCGCTCTGATTAATCCAGTTTTCACACCCAATGT
CGGTCGATACGATCCAGTTACGCACATCTGGAGCGGACTCAATCTGGCAAGTG
AGCAAAGCATTTTCATGACGCTCACAGGCACAATCAACTCAACGACGACCGGTA
TTTTGCTGAACACGGTCAGCGTAAGGCCACCTGCGGACACCACCGACCCGAAC
GCATTGGATAACAGCGCTACCGACGCGACCTTGATCGAAGCTCGGGCCT***GTGG***
AGGTCGCTAGATGGTC

Appendix iv

Whole genome library prepared from hRSV RNA (tail primer in bold and italic)

>hRSV-Clone-1-hRSV-PartialPositive-18bp-HumanChrom-15-Full

GACCATCTAGCGACCTCCACAGCCCCACCAGCAGTGTATGATGTTTCCAGTGT
CTCATACCTTCACCAACACTTGGTATTGACAGACATTTTTATCCTTTACCAGTGT
CAGGGTATAAAATTATATCTTGTTGACTTAAATTGCATTTATTTGGTCACTAGTGA
AGTCAAGCATAATTTTATATGTTTATTGGTCATTTGGGTTTCCTTTTGAATTGCCT
AGTCACATCCTTCCTTTCTTTTTCTTGCAG***GTGGAGGTCGCTAGATGGTC***

>hRSV-Clone-2-HumanChro-17

gACCATctaGCGAcCTCCACCCACCAAGtTACAGAAGAGGATTAGCACCtTGCTCT
CACtgtgGAGAGAGAGGGAGAATCGCTGGCCCTCACGGGCTGCCAGGCGTGGG
GAGGCCGACAGACCCAGTGGATGAGCTAGAAGGGAGAGAATGACATTTGTTCA
TATTCCTAAAGGAGCTGCTTTCCTGGGTCAGAGCCTTAGCTTCTCTAGTCCCTC
TTTCTCTTTGAGCATCTATTAACCACCAATGGGGGGCCACTAGAGGGCCT***GTGG
AGGTCGCTAGATGGTC***

>hRSV-Clone-3-HumanChro-17

GACCATctaGCGACCTCCACATGCGAAACACATTgtAGGAGCTCAGAAGATGTCA
GCAATAGTTTTTATCTTACCACTATCACTAATGTTACTACCATTGATGTTATTCTC
ATCATCATTATAATTATCTcTGAGTGCTCAGCATCTAGTAGATGCTTGGTAAATAT
CAAATCAGTGGATGAATGACTACCTCTAGAAGCTCCTTTTATTCCTTCATTACAG
GTCCTGGCTCCCTGACTTCATTTCTCCTATATATTTTATGCAGGATCTGCTTCAT
GGCAGAACAGAGTAATGGTTAAGAGCTGGG***GTGGAGGTCGCTAGATGGTC***

>hRSV-Clone-4-HumanChro-17

GACCATCTAGCGACCTCCACATGCGAAACACATTGTAGGAGCTCAGAAGATGT
CAGCAATAGTTTTTATCTTACCACTATCACTAATGTTACTACCATTGATGTTATTC
TCATCATCATTATAATTATCTCTGAGTGCTCAGCATCTAGTAGATGCTTGGTAAA
TATCAAATCAGTGGATGAATGACTACCTCTAGAAGCTCCTTTTATTCCTTCATTA
CAGGTCCTGGCTCCCTGACTTCATTTCTCCTATATATTTTATGCAGGATCTGCTT
CATGGCAGAACAGAGTAATGGTTAAGAGCTGGG***GTGGAGGTCGCTAGATGGT
C***

>hRSV-Clone-5-HumanChro-17

GACCATCTAGCGACCTCCACCCACCAAGTTACAGAAGAGGATTAGCACCTTGC
TCTCACTGTGGAGAGAGAGGGAGAATCGCTGGCCCTCACGGGCTGCCAGGCG
TGGGGAGGCCGACAGACCCAGTGGATGAGCTAGAAGGGAGAGAATGACATTT
GTTCATATTCCTAAAGGAGCTGCTTTCCTGGGTCAGAGCCTTAGCTTCTCTAGT
CCCTCTTTCTCTTTGAGCATCTATTAACCACCAATGGGGGGCCACTAGAGGGCC
T***GTGGAGGTCGCTAGATGGTC***

>hRSV-Clone-6-Partially-Matched-hRSV-18bp-**HumanChrom-14-Full**

GACCATCTAGCGACCTCCACCGCCCTTAATCCATTTAACCCTGAGTTGACACA
GCACAtgTTTCAGAGAGCACGGGCTTGGGGGTAAGGTTATAGATTAACAGCATC
CCAAGGCAGAAAAATTTTTCGTAGTACAGAACAAAATGGAGTCTCCTATGTTTAC
TTCTTTCTACCCAGACACAGTAACAATCTGATCTCTCTGTCTTTTCCCCACATTC
CCCCTTTTCTTTTCGACAAAACCGCCATTGTCATCATGGCCCATTCTCGATGGT
CGCTTTCTCTT***GTGGAGGTCGCTAGATGGTC***

>hRSV-Clone7-PartMatched-hRSV-18bp-Full-HumanChrom-14-Full

GACCATCTAGCGACCTCCACCGCCCTTAATCCATTTAACCCTGAGTTGACACA
GCACATGTTTCAGAGAGCACGGGCTTGGGGGTAAGGTTATAGATTAACAGCAT
CCCAAGGCAGAAAAATTTTTCGTAGTACAGAACAAAATGGAGTCTCCTATGTTTA
CTTCTTTCTACCCAGACACAGTAACAATCTGATCTCTCTGTCTTTTCCCCACATT
CCCCCTTTTCTTTTCGACAAAACCGCCATTGTCATCATGGCCCATTCTCGATGG
TCGCTTTCTCTT***GTGGAGGTCGCTAGATGGTC***

>hRSV-Clone-8-PartMatched-hRSV-18bp-HumanChrom-1-9-HumanChrom-14

GACCATCTAGCGACCTCCACCGCCCTTAATCCATTTAACCCtgaGTTGACACAG
CACAtgTTTCAGAGAGCACGGGCTTGGGGGTAAGGTTATAGATTAACAGCATCC
CAAGGCAGAAAAATTTTTCGTAGTACAGAACAAAATGGAGTCTCCTATGTTTACT
TCTTTCTACCCAGACACAGTAACAATCTGATCTCTCTGTCTTTTCCCCACATTCC
CCCTTTTCTTTTCGACAAAACCGCCATTGTCATCATGGCCCATTCTCGATGGTC
GCTTTCTCTT***GTGGAGGTCGCTAGATGGTC***

>hRSV-Clone-9-HumanChrom8-Full

GACCATCTAGCGACCTCCACCAGACAAAAACAGGACCGCACGATGGAATGAGA
GAGCTGCAGTGACAGGGGGACGTTGAGGCAGGCAGAGGTGAGAAGCAGTGAG
AACGAAGGGAATGCTGGGTGCATCCTGAGGAAGCCTCTCCCATCTAGAAGCCA
CCAGGACTATCGCAGGCGGGCTATGAAACACCAGGCTTTGGAGCAAGATGACT
GTGTCTCTCCCAAAAGGCTTACAGAAGCAAAATTGAGTTCGT***GTGGAGGTCGCT
AGATGGTC***

>hRSV-Clone-10-HumanCrhom-8-Full

GACCATCTAGCGACCTCCACCAGACAAAAACAGGACCGCACGATGGAATGAGA
GAGCTGCAGTGACAGGGGGacGTTGAGGCAGGCAGAGGTGAGAAGCAGTGAG
AACGAAGGGAATGCTGGGTGCATCCTGAGGAAGCCTCTCCCATCTAGAAGCCA
CCAGGACTATCGCAGGCGGGCTATGAAACACCAGGCTTTGGAGCAAGATGACT
GTGTCTCTCCCAAAAGGCTTACAGAAGCAAAATTGAGTTCGT***GTGGAGGTCGcT
AGATGGTC***

Poster presented at the postgraduate fare, school of life sciences, University of Westminster in April, 2009.

UNIVERSITY OF WESTMINSTER

Validation and adaptation of metagenomic approaches to identify novel viruses in patients with upper respiratory tract infection

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Introduction

Metagenomics is a very new field that helps to study the genomic diversity in the environment, bypassing the conventional culture techniques which obviously have limitations to grow all of the members present in nature. In other words it is a culture independent or sequence independent approach to isolate and identify new microorganisms directly from any environmental or clinical sample. Metagenomic approaches to viral characterisation have been applied to seawater, near shore sediments, faeces, serum, plasma and respiratory secretions and have broadened the range of known viral diversity (Allender *et. al.*, 2001; Breitbart *et. al.*, 2003 &2002; Mushahwar *et al.*, 1999). Selection of samples with high viral loads, purification of viral particles, removal of cellular nucleic acids, efficient sequence-independent amplification of viral RNA and DNA, recognisable sequence similarities to known viral sequences and deep sampling of the nucleic acid populations through large scale sequencing can all improve the ability to detect new viruses. Viral metagenomic approaches provide novel opportunities to generate an unbiased characterisation of the viral populations in various organisms and environments.

The current study will be carried out to explore the possible use of the sequence-independent single primer amplification (SISPA), phi29 DNA polymerase based amplification and other related methods to study viral diversity and the possible presence of new viruses in respiratory infections. The use of phiX29 DNA polymerase provides highly uniform amplification across the entire genome, with negligible sequence bias. This technique is based on multiple displacement amplification (MDA) technology which carries out isothermal genome amplification using phiX29 DNA polymerase replicating upto 100 kb without dissociating from the genomic DNA template. The DNA polymerase has 3'->5' exonuclease proofreading activity to maintain high fidelity during replication and is used in the presence of exonuclease resistant primers to achieve high yield of DNA product.

Objectives

1) Optimisation of novel metagenomic methods using phi 29 DNA polymerase and sequence independent amplification with known quantities of viruses e.g. bacteriophages λ and MS2 as control viruses.

2) Validation and adaptation of sequence-independent and phi 29 DNA polymerase based amplification to improve the yield and detection of viruses from volunteer nasal samples.

3) Application of optimised methods using environmental or clinical samples.

4) Enrichment, cloning and sequencing of novel amplified products from above approaches.

5) Examination of the subcloned and sequenced products for similarities to known viruses using BLASTn, tBLASTx and discovery of possible new virus(es) in the studied samples.

Figure1: General strategy to look for virus population in viral metagenome

Methods

Samples of nasal secretions will be obtained from patients with uncomplicated acute respiratory disease from whom no aetiological agent has been found using standard laboratory methods. Nasal secretions from asymptomatic volunteers will serve as control samples and used to study viral diversity in healthy individuals. Sample will be processed by centrifugation to remove cellular materials; the supernatant, containing free viral particles will be treated with DNase and RNase to remove residual nucleic acids. Viral nucleic acid, protected from nuclease digestion will be extracted (Figure 1). In order to include viruses with RNA and single stranded DNA genomes in the analysis, a portion of the sample will be subjected to reverse transcription using random primers and 2nd strand synthesis reaction to give double stranded DNA product. The DNA will then be screened by PCR for a series of known viral pathogens responsible for acute respiratory tract infection (ARI). Patients from whose samples viral pathogens are detected will be excluded from the study. From the remainder, the DNA extracts will be amplified by phi29 DNA polymerase and/or linkers to produce shotgun libraries. Amplified DNA will be selectively enriched for certain groups of virus for cloning. The cloned inserts will be sequenced and the sequence information subjected to bioinformatics analysis aimed at identifying the composition and overall diversity of virus populations present in healthy volunteers and in patients with ARI.

Results

In the preliminary study of this project bacteriophage lambda was used as a prototype of DNA virus to evaluate several techniques. Different concentrations of this virus were prepared in buffer solution to assay DNase protection, DNA extraction and subsequent detection by PCR. It was found that, virus particles were protected by their envelop after DNase treatment (1U DNase / 8µl of sample at 37°C), which was further confirmed by PCR (Figure 2). Whole genome of bacteriophage lambda was amplified by phi 29 DNA polymerase (Fig 4) which ultimately increased the sensitivity of detection of lambda DNA down to 10^3 pfu/ml as confirmed by PCR (Fig 3). A protocol was also evaluated for preparation of standard curve with Quant-iT PicoGreen DNA assay kit to determine concentrations of DNA up to 25pg (data not shown). A recombinant plasmid was prepared using commercial vector pCR2.1 having an insert of 500bp of bacteriophage lambda DNA. This recombinant plasmid has been used in qPCR to construct a standard curve for absolute quantification of virus particle in future studies.

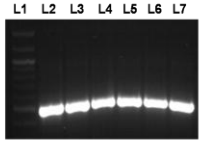

Figure 2: Virus particles were detected after DNase treatment (1U DNase / 8µl of sample at 37°C up to 3 hrs. L1, 100bp DNA ladder; L2, 30 min; L3, 1hr; L4, 1.5 hr; L5, 2hrs; L6, 2.5 hrs; L7, 3hrs

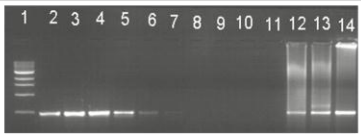

Figure 3: DNA extraction from different concentration of phage particle by QIAamp DNA mini kit and detection by PCR before and after phi 29 DNA amplification. Virus particles were detected up to 10^3 pfu/ml by PCR after phi 29 amplification. Lane1, 100bp DNA ladder; Lane2, 10^{10}; Lane3, 10^8; Lane4, 10^6; Lane5, 10^7; Lane6, 10^6; Lane7, 10^5; Lane8, 10^4; Lane9, 10^3; Lane10, 10^2 pfu/ml before phi29 amplification & Lane12, 10^5; Lane13, 10^4; Lane14,10^3 pfu/ml after phi 29 amplification.

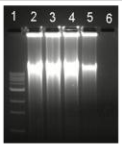

Figure 4: Amplification of lower concentrations of bacteriophage lambda DNA by phi 29 DNA polymerase in the 2nd round. Lane1, 1kb DNA ladder; Lane2, *E. coli* DNA as a control; Lane3, 10^4 pfu/ml; Lane4, 10^3 pfu/ml; Lane5, 10^2 pfu/ml; Lane6, 10^1pfu/ml.

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Poster presented at conference organized by the Society of General Microbiology in spring, 2010

UNIVERSITY OF FORWARD THINKING WESTMINSTER

Use of ligation to enhance phi 29 DNA polymerase-mediated amplification and detection of viral cDNA

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Introduction:

Phi29 DNA polymerase is an enzyme that efficiently amplifies various types of DNA with strand displacement capacity[1-3]. Phi29 DNA polymerase is derived from *Bacillus subtilis* phage phi29 and used for whole genome amplification. The amount of double stranded DNA can be amplified more >1000 fold using phi29 DNA polymerase, when it is highly purified and >2kb in length[4]. It has been found in previous studies that ligation of double stranded smaller genomes significantly improved the amplification of DNA after phi29 amplification[4]. Reverse transcription of viral RNA generates different sizes of cDNA, which are usually smaller than that of virus genomes. Using such shorter fragments of cDNA for phi29 DNA polymerase amplification results in inefficient amplification. In a recent study Berthet *et al.*, (2008) amplified ligated viral cDNA using phi29 DNA polymerase and found a significant improvement in the amplification of viral cDNA. A more robust protocol needs to be developed and optimised in order to improve amplification of single stranded DNA e.g. viral cDNA using phi29 DNA polymerase. This study was conducted to optimise the amplification of a small virus genome (MS2) to include a ligation step prior to phi29 DNA polymerase amplification.

Materials and Methods:

MS2 RNA (Roche, Burgess Hill, Germany) was used to prepare cDNA using the 1st strand synthesis kit (NEB, Hitchin, UK) for a ligation assay. The ligation assay was done in two ways, ligating single stranded (ss) cDNA using T4 RNA ligase and double stranded (ds) cDNA using T4 DNA ligase from Fermentas, York, UK (Figure 2). Both ligated ss and ds cDNA were amplified using phi29 DNA polymerase (Qiagen, Crawley, UK).

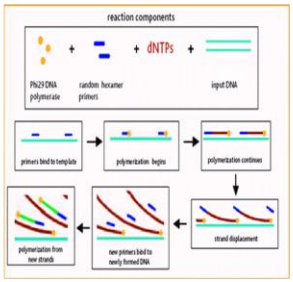

Figure 1: Schematic diagram of whole genome amplification with phi29 DNA polymerase. Source: http://www.biocompare.com.

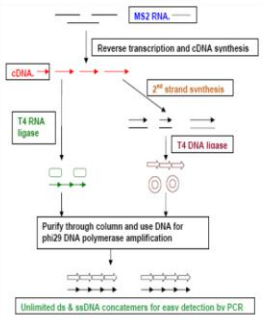

Figure 2: Application of T4 RNA ligase & T4 DNA ligase to improve detection of viral RNA. cDNA (→), circularised ssDNA (▭), ligated ssDNA (→→→), ligated dscDNA (⇒⇒⇒), circularised dscDNA (◎).

Amplified DNA was diluted (1:20) in 10 mM Tris-HCl (pH 8.0) to use them as template (5 µl) for PCR detection using HotStarTaq DNA polymerase (QIAGEN, Crawley, UK). PCR was started with an activation of 15 minutes at 95°C, followed by 35 cycles having 94°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute. A final extension was done at 72°C for 10 minutes.

Results:

Double stranded blunted MS2 cDNA was prepared from a stock of 10^8 copy/µl and divided into two aliquots, one was ligated with T4 DNA ligase and the other was not ligated. Both of these ligated and non-ligated dscDNA were diluted from 10^6 to 10^3 copy/µl and detected by PCR. Both of these sets of dilutions were further amplified using phi29 DNA polymerase and consequently detected by PCR (Figure 3).

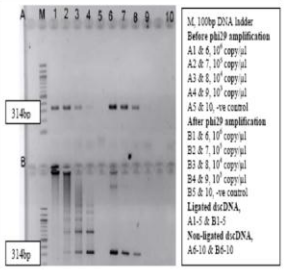

Figure 3: PCR detection of MS2 dscDNA, ligated (A1-4 and B1-4) with T4 DNA ligase and non-ligated (A6-9 and B6-9) forms, before (A1-10) and after (B1-10) phi29 DNA polymerase (REPLig) amplification. DNA ladder (100bp); NEB, Hitchin, UK.

It was found that phi29 DNA polymerase mediated amplification of dscDNA improved the detection of the ligated products more than the non-ligated products. In order to determine the amount of amplification of dscDNA in Figure 3 (B1-4), this assay was repeated. Double stranded cDNA (MS2) 10^8 copy/µl was ligated with T4 DNA ligase, diluted up to 10^5 copy/µl and amplified using phi29 DNA polymerase. A control was run besides without any ligation of dscDNA and amplified by phi29 DNA polymerase. Amplified DNA from both the ligated and non-ligated dscDNA were serially diluted and subsequently detected by PCR (Figure 4). Ligated double stranded MS2 cDNA was amplified more than 10^5 times compared to the non-ligated product.

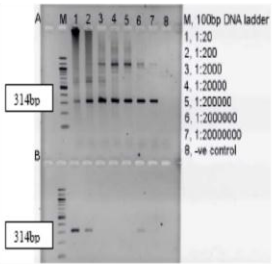

Figure 4: PCR detection of ligated (A1-7) and non-ligated (B1-7) dscDNA of MS2 bacteriophage after phi29 (REPLig) amplification. DNA ladder (100bp); NEB, Hitchin, UK.

In another study, T4 RNA ligase assay was done in two ways, adding MS2 RNA (10^7 copy/µl) to total RNA, 50 ng/µl, (NEB, Hitchin, UK) to protect MS2 RNA from degradation and without adding any total RNA. The MS2 RNA with and without total RNA were further diluted to 10^3 copy/µl and reverse transcribed to cDNA using 5'-phosphorylated random hexamers. All of these dilutions of cDNA were ligated with T4 RNA ligase, purified through a DNA purification column and amplified with phi29 DNA polymerase for subsequent detection by PCR (Figure 5). T4 RNA ligase was shown to improve the detection of MS2 RNA 10-fold in the presence of background RNA.

Table 1: Summary of Results

Nucleic acids type	Phi29 amplification improved PCR detection
ds MS2 cDNA	>10^5 fold
MS2 cDNA in the presence of total cDNA	10 fold

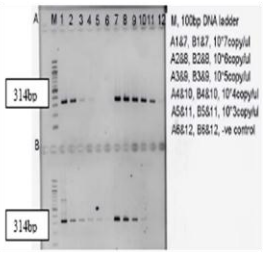

Figure 5: PCR detection of sscDNA of MS2 with 50ng/µl total RNA (A1-5 & B 1-5) and without total RNA (A7-11 & B7-11), ligated with T4 RNA ligase, before (A1-12) and after (B1-12) phi29 DNA polymerase amplification. DNA ladder (100bp); NEB, Hitchin, UK.

Discussion:

Unlike phi29 amplification of dsDNA viral RNA requires modifications before it can be amplified in this way. We have clearly shown that ligation of ss and ds cDNA significantly improved phi29 amplification (Table 1). This study supports the findings of previous studies on viral RNA[2,4].

Our earlier studies showed that single stranded cDNA could not be amplified using phi29 DNA polymerase without any modifications (data not shown). In this study total RNA was added to MS2 RNA to protect it from degradation in case of very low concentration of target RNA. The other reason of adding total RNA was to create molecular crowding in order to improve efficiency of ligation and amplification. Prior ligation of cDNA has been shown by us and others to improve amplification by phi29 DNA polymerase and can occur even at low copy number[1]. This may have beneficial effects in enhancing the sensitivity of virus detection from clinical samples.

It may be concluded that, viral RNA can be significantly amplified using phi29 DNA polymerase, from both ss and ds cDNA, using our modified protocol (Figure 2). Further research needs to be done to fully optimise this procedure.

References

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Acknowledgement:

We would like to thank the SGM for providing a bursary to MSK to attend this meeting. We are also indebted to the University of Westminster to support MSK with Cavendish scholarship for this research.

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