Detection of Bacteriophage and Respiratory Viruses in Droplets

by

Phillipa Perrott

Bachelor of Applied Science (Honours)

A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

School of Life Sciences, Faculty of Science and Technology

Queensland University of Technology

2011

Keywords

Influenza, respiratory viruses, bacteriophage, aerosols, airborne transmission, nested real-time PCR

Abstract

Influenza is a widespread disease occurring in seasonal epidemics, and each year is responsible for up to 500,000 deaths worldwide. Influenza can develop into strains which cause severe symptoms and high mortality rates, and could potentially reach pandemic status if the virus' properties allow easy transmission. Influenza is transmissible via contact with the virus, either directly (infected people) or indirectly (contaminated objects); via reception of large droplets over short distances (one metre or less); or through inhalation of aerosols containing the virus expelled by infected individuals during respiratory activities, that can remain suspended in the air and travel distances of more than one metre (the aerosol route). Aerosol transmission of viruses involves three stages: production of the droplets containing viruses; transport of the droplets and ability of a virus to remain intact and infectious; and reception of the droplets (via inhalation). Our understanding of the transmission of influenza viruses via the aerosol route is poor, and thus our ability to prevent a widespread outbreak is limited. This study explored the fate of viruses in droplets by investigating the effects of some physical factors on the recovery of both a bacteriophage model and influenza virus. Experiments simulating respiratory droplets were carried out using different types of droplets, generated from a commonly used water-like matrix, and also from an 'artificial mucous' matrix which was used to more closely resemble respiratory fluids. To detect viruses in droplets, we used the traditional plaque assay techniques, and also a sensitive, quantitative PCR assay specifically developed for this study. Our results showed that the artificial mucous suspension enhanced the recovery of infectious bacteriophage. We were able to report detection limits of infectious bacteriophage (no bacteriophage was detected by the plaque assay when aerosolised from a suspension of 10^3 PFU/mL, for three of the four droplet types tested), and that bacteriophage could remain infectious in suspended droplets for up to 20 minutes. We also showed that the nested real-time PCR assay was able to detect the presence of bacteriophage RNA where the plaque assay could not detect any intact particles. Finally, when applying knowledge from the bacteriophage experiments, we reported the quantitative recoveries of influenza viruses in droplets, which were more consistent and stable than we had anticipated. Influenza viruses can be detected up to 20 minutes (after aerosolisation) in suspended aerosols and possibly beyond. It also was detectable from nebulising suspensions with relatively low concentrations of viruses.

Contents

List of Figures

List of Tables

List of Supplementary Materials

Disc containing raw data

Appendix A: Original Article

P. Perrott, G. Smith, Z. Ristovski, R. Harding, M. Hargreaves (2009). A nested real-time PCR assay has an increased sensitivity suitable for detection of viruses in aerosol studies.

J. Appl. Micro., **106**, 1438-1447.

Appendix B: Synthetic control sequences

List of Abbreviations

°C	degrees celcius
µL	microlitre/s
µm	micrometre/s
ng	nanogram/s
pmol	picomole/s
L/min	litres per minute
ATP	adenosine tri phosphate
bp	base pairs
cDNA	complementary DNA
CFU	colony forming unit
cm	centimetre/s
CMD	count median diameter
CPE	cytopathic effects
DNA	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetate
g	gram/s
h	hour/s
HA	haemagglutinin
HPAIV	highly pathogenic avian influenza virus
ISO-CEN	Cooperation of the International Organization for Standardization and the European Committee for Standardisation
L	litre/s
LRT	lower respiratory tract

mL	millilitre/s
min	minute/s
NA	neuraminidase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming units
PIV1	parainfluenza virus subtype 1
PNK	polynucleotide kinase
QFSS	Queensland Forensic Scientific Services
RH	relative humidity
RNA	ribonucleic acid
rpm	rotations per minute
RSV	human respiratory syncytial virus
RT-PCR	reverse transcriptase polymerase chain reaction
s	second/s
SARS	severe acute respiratory syndrome
ssRNA	single-stranded ribonucleic acid
TASA	tryptone agar (with) streptomycin and ampicillin
TBSA	tryptone broth (with) streptomycin and ampicillin
$TCID_{50}$	tissue culture infective dose 50 %
U	unit
URT	upper respiratory tract
UV	ultra violet
WHO	World Health Organisation
w/v	weight/volume

Statement of Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature

Date

Acknowledgements

I would like to thank the staff at Queensland Health Forensic and Scientific Services, particularly the staff in the Public Health Virology department, who offered me so much help in the laboratory. Special thanks to Judy Northill, Alyssa Pyke, Bruce Harrower, and Andrew Day, each of whom went out of their way when I needed help. I would also like to thank all of my friends at Queensland Health Forensic and Scientific Services, just for being there and sharing the experience of being a research student.

Thanks to staff and students at the International Laboratory for Air Quality and Health, in particular Rob Modini, Graham Johnson and Luke Knibbs who helped me a lot with the air testing equipment. Thanks also to my friends at QUT for all their support, especially to the Environmental Microbiology Research Group, who have always been very supportive and friendly. In particular, thanks to Flavia Huygens and Megan Hargreaves, who are always positive, resourceful and supportive in all facets of research life. Thanks to each of my supervisors, but especially to my principal supervisor Megan Hargreaves, who has provided me so many opportunities over the years, for which I am very grateful.

Lastly I would like to thank my dear friends and family for their never-ending support, not only in the completion of my PhD, but in all endeavours which I have chosen to pursue. Thanks to the Perrott family, the Lord family and the Young and Crosbie families, who have enthusiastically supported me along the way. To my dad Bruce and my sister Eloise, who have endured my lengthy university vocation, and without whom I would have never got this far, thank you for everything. Thanks to my boyfriend Harley, who has been by my side since the beginning of this PhD, and has suffered the highs and the lows of the experience with me. His unwavering support has seen me through to the end.

Lastly, to my dear mum Jenny - despite her passing in my first year of university, I would never have reached this point without her. This thesis is dedicated to her memory.

Chapter 1: Introduction and Literature Review

1.1 Introduction

Influenza is an acute respiratory disease, causing upper respiratory tract infections in 5-15% of the world's population each annual seasonal epidemic (World Health Organisation, 2010b). The economic costs associated with illnesses like influenza and the common cold are in the order of billions of dollars, arising from medical treatment, lost income and decreased productivity of ill workers. Influenza can be more severe for the young, elderly or immuno-compromised, causing complications and death in many cases. Severe influenza illness is believed to be responsible for up to 500,000 deaths worldwide per year (World Health Organisation, 2009).

The Centers for Disease Control (CDC) states that the most common mode of transmission of influenza is by 'large-particle respiratory droplet transmission', which is short-range transmission of droplets to susceptible people, over a distance of up to one metre (Fiore *et al.*, 2008). Influenza can also be spread by direct contact with an infected person, or by indirect contact with an intermediate contaminated object (fomite) (Goldmann, 2000). However, the mode of transmission of which the least information is available, is airborne transmission. This occurs when droplets evaporate and become droplet nuclei, which are small residue droplets with diameters of less than 5 μm, that can remain suspended in the air for long periods of time (Fiegel *et al.*, 2006). Droplet nuclei are thus associated with long distance transmission.

Virus integrity and spatial distribution of droplets are dependent on a number of factors, including droplet size and atmospheric conditions. Our knowledge of the mechanisms by which viruses are released into the air by humans or animals is somewhat limited, as is the nature of the association between the virus and its carrier particles. Furthermore, understanding of the mechanisms responsible for transport and spread of the agents in common types of indoor environments such as residential apartments, buildings, hotels and hospitals is also very limited.

While studies have been conducted to characterise the distribution of such virus-containing droplets, results have varied widely. Most of these studies have used models to simulate natural aerosolisation, and until recently, no studies had examined the natural cough droplet distribution of infected human subjects.

A greater understanding of the fate of viruses in droplets, and the effect of physical factors that govern aerosol spread, is needed to control the spread of viral infections. The research undertaken in this thesis addresses some questions surrounding aspects of virus transmission via the aerosol route, in order to contribute to a more comprehensive understanding of this issue.

1.2 Literature review

1.2.1 Influenza and other respiratory infections

Influenza is a rapidly evolving virus, and is widespread throughout the world. Influenza disease occurs mainly during the winter season, from March to August in Australia, in seasonal epidemics known as the 'flu season'. There are three types of influenza virus: types A, B and C. These differing types are based on the antigenic differences in the nucleoprotein and matrix proteins, which influence the host-type (Schweiger *et al.*, 2000). Influenza A is found in humans, swine, birds, horses, sea mammals and other animals, whereas influenza B and C infect only human hosts.

Influenza is an enveloped, negative-sense, single stranded RNA (ssRNA) virus with a diameter of about 80-120 nm, and whilst it is pleomorphic, it tends to maintain a generally spherical shape. The influenza virus genome consists of eight linear segments of ssRNA, with 11 genes encoding for 11 proteins; the entire genome is approximately 12,000 bp in size. It is this segmentation of the virus that endows it with its relatively high mutation rate of about $10^{-4.5}$ mutations per replication (Holland *et al.*, 1982).

Embedded in the lipid bilayer envelope are two glycoproteins: haemagglutinin (HA) and neuraminidase (NA) (Wiley and Skehel, 1987) (see fig 1-1). HA is responsible for fusion of the cell membranes to viral membranes, and also for binding of the virus to the sialic acid-containing cell receptors (Bullough *et al.*, 1994). NA frees the virus particle from host cell receptors, by cleaving the terminal sialic acid from the glycoproteins, thereby facilitating virus spread (Webster *et al.*, 1992). Influenza A is further classified into subtypes, by the different combinations of HA and NA types it possesses: sixteen HA subtypes and nine NA subtypes exist (Schweiger *et al.*, 2000). However, only HA 1, 2 and 3 and NA 1 and 2 commonly exist in humans (Lynch and Walsh, 2007). The most common strains observed in seasonal epidemics are H3N2 and H1N1.

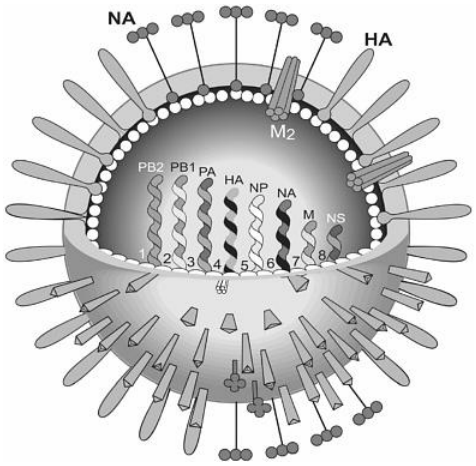


Figure 1-1 Structure of influenza virus (taken from "Microbial Threats to Health: The Threat of Pandemic Influenza")

Most of the evolutionary changes in influenza viruses occur in the surface glycoproteins (HA and NA), as a result of host immunological pressure (Webster *et al.*, 1992). Two major mechanisms of genetic evolution in influenza viruses are antigenic drift and antigenic shift, which cause epidemics (increased occurrence of a disease amongst people in a community or region, in a given period of time) and pandemics (spread of disease over a large geographical area, spanning several countries or the world), respectively. Annual epidemics of influenza A throughout most parts of the world are due to antigenic drift, which occurs when the HA or NA subunits mutate. Pandemics are due to antigenic shift, which is the reassortment of RNA segments between different influenza A subtypes in a single host cell (Schweiger *et al.*, 2000). This occurs in a host which becomes infected with two different strains. The strains can then swap their genetic material (reassort) to create a new strain. This is very important for the evolution of influenza A as it has many gene pools (host-types) in the environment. Reassortment has been demonstrated in influenza B in a laboratory setting, however, this is not considered important as the only known gene pool is in humans (Webster *et al.*, 1992).

Three major pandemics occurred in the 20th century, in 1918 (Spanish flu), 1957 (Asian flu) and 1968 (Hong Kong flu). The Spanish flu, an H1N1 strain, took place at the time of World War 1, in 1918, and was responsible for the deaths of millions of people around the world. In particular, this strain infected young, healthy men, including many soldiers. Asian flu was an H2N2 subtype, and Hong Kong flu was an H3N2 subtype. These two epidemics were better known for the infection of infants, the elderly, and the immunocompromised (Hsieh *et al.*, 2006).

More recently, we have seen two further strains of influenza come to prominence in public health. The first of these was an avian influenza strain known as highly pathogenic avian influenza virus (HPAIV). The virus was first observed in 1997 in Hong Kong, where it crossed the human-avian species barrier (Claas *et al.*, 1998). It infected eighteen people, resulting in six fatalities (Yuen *et al.*, 1998), before re-emerging among humans in 2003. In 2006, it bordered on pandemic status; however it did not spread efficiently enough between humans for it to be declared pandemic by the World Health Organization (WHO). Although four avian influenza subtypes have been observed in humans, this recent strain is considered the greatest threat to human health of the avian strains, as the symptoms are more severe, and it has a high mortality rate of about 60 % (Korteweg and Gu, 2008). The spread of HPAIV was mainly via direct contact with infected birds (World Health Organisation, 2006).

The second recent strain of influenza virus was a strain of swine influenza (H1N1), which spread globally, and was proclaimed pandemic by the WHO in 2009. As of August 1, 2010, it had spread to more than 214 countries and was responsible for 18,449 reported deaths, 1,992 of which occurred in South East Asia. In early August 2010, it was declared to be in a post-pandemic state (World Health Organization, 2010). Whilst swine flu did not exhibit the severity of disease that HPAIV did, its spread among humans was far superior to that of HPAIV.

Influenza surveillance systems have been in place since 1952, in response to the global health threats posed by influenza viruses. They monitor strains in circulation that cause epidemics by collating data gathered from infected people on both a regional and a global scale. The aim is to monitor influenza evolution, and to predict and prevent the occurrence of another pandemic such as those endured in the past. The data is also used to develop annual influenza vaccinations. Australian influenza epidemiology data is published in the Annual report of the National Influenza Surveillance Scheme.

Kaczmarek and colleagues (2010) published the most recent Annual report of the National Influenza Surveillance Scheme, and compared the incidence of laboratory-confirmed influenza illnesses from 2004-2008 (see figure 1-2). They reported that the levels of influenza were fairly mild and stable from 2004-2006, but in 2007 there was a large increase in influenza infections. The reason for this was not clear, and in 2008 influenza levels dropped slightly, though remained above average. Although more recent comparative data has not yet been published, it can be assumed that the flu seasons of 2009 and 2010 were also high above average, due to the prolificacy of swine flu.

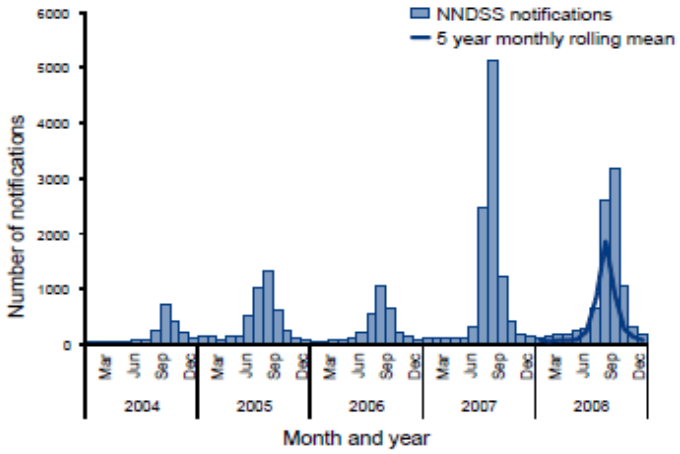


Figure 1-2 Laboratory-confirmed influenza notifications in Australia, 2004-2008. Figure taken from (Kaczmarek *et al.*, 2010)

Given our geographical location, the frequencies of different strains of influenza A viruses are not always in accordance with those in other areas of the world. For example, in 1995, the predominant influenza strain around the world was influenza A (H3N2), however, the most frequently isolated strain in Australia was influenza A (H1N1), the incidence of which increased from previous flu seasons (Anonymous, 1995). In 2003, the predominant strain was H3N2. This strain was closely related to a Fujian strain, A/Fujian/411/2002 (H3N2), and significant antigenic drift was observed (Yohannes *et al.*, 2004). In 2004, illnesses caused by influenza viruses were 41% lower than in 2003, and influenza A (H3N2) was once again the predominant strain (Li *et al.*, 2005a). Influenza A H3N2 viruses remained the predominant strain in Australia up to 2007. However, Kaczmarek and colleagues (2010) reported that in 2008, there was a very high proportion of influenza B isolates: 66% of influenza viruses analysed by WHO were influenza B, whilst only 21.7% were influenza A H3N2 and 11.8% were influenza A H1N1. Also in this year, 41% of the national influenza notifications occurred in Queensland, which is surprisingly high, compared to 20% in New South Wales and 14% in Victoria.

In 2010, influenza A (H1N1) was again the most frequent strain. Influenza surveillance data reported to the Australian Health Departments up to 5 November 2010, shows that of 11,317 positive influenza cases, 60% of these were pandemic swine flu H1N1; 29% was influenza A (untyped); 2% was influenza A H3N2; 7% was influenza B; and the remaining 2% were untyped (Australian Government Department of Health and Ageing, 2010).

Two other medically important viruses are parainfluenza viruses (PIV) and respiratory syncytial viruses (RSV), which are the two major causes of lower respiratory tract (LRT) infections in infants and young children. More recently, these two viruses have also been recognised as important pathogens in adults, although the symptoms are less distinct and often the viral cause is not initially identified correctly (Hall, 2001). Additionally, there are no effective means of controlling the spread of these two viruses.

There are four types of PIV that occur in humans: serotypes PIV-1, PIV-2, PIV-3 and PIV-4. Serotype PIV-3 is the most commonly associated with severe LRT infections, whereas PIV-4 is the least associated of the four types (Echevarria *et al.*, 1998). There are two subtypes of RSV, serotypes A and B (Liolios *et al.*, 2001). RSV is responsible for approximately 90,000 hospitalisations and 4,500 deaths in children aged six months and younger of the same age group, each year in the US. Stockton and colleagues stated that although the incidence of RSV in infants is known and widely documented, its contribution to morbidity and mortality in adults is unknown, and possibly under-diagnosed (Stockton *et al.*, 1998).

There are three recognised mechanisms by which respiratory viruses can spread: contact, either directly with an infected person or animal, or indirectly by contact with a contaminated object (fomite); by droplet transmission, where large droplets are expelled by an infected host and come into contact with the conjunctiva or mucous membranes of a potential host; or by the airborne route, via small droplets (droplet nuclei) that can remain suspended in the air for long periods of time (i.e. at least several minutes) (Garner, 1996, Nicas *et al.*, 2005). Some consider infectious droplets, known as bioaerosols, to be the principal mode of transmission (Couch *et al.*, 1986); others believe that direct and indirect contact is more to blame for spread of the disease. This issue remains contentious, with support on both sides, and evidence varying widely. However, it is generally agreed by most experts that airborne transmission of viruses is an issue requiring further investigation. As methods for droplet measurement and virus detection continue to become more sophisticated, more data is becoming available.

1.2.2 Bioaerosols

Bioaerosols have been defined as a collection of aerosolised biological particles (Cox and Wathes, 1995), which can vary depending on source, dispersal mechanisms and environmental conditions (Pillai, 2002). Bioaerosols can be pathogenic themselves, or can act as a vehicle for the dissemination of pathogens (Pillai and Ricke, 2002), such as fungi, bacteria or viruses. It is thought that many infections, particularly respiratory infections,

can be transmitted between persons via airborne droplets from an infected person to an uninfected person. These droplets, or infectious aerosols, contain pathogens, which upon contact or inhalation may cause disease in a susceptible person. Therefore, bioaerosols contribute substantially to the transmission of many infectious diseases. Researchers, however, are unable to reach a consensus as to the significance of the airborne route in the transmission of respiratory diseases including influenza, due to a shortage of evidence for either argument. This can be attributed to past inadequate technologies, which were unable to fully recognise all facets of bioaerosol distribution. However, even with the aid of the more sophisticated technology that has become available over the last couple of decades, concurrence among experts has still not been attained.

Infectious bioaerosols can be generated from a number of sources, including sewage treatment plants (Carducci *et al.*, 1999), toilets (Barker and Jones, 2005), and infected people. In this study, we are only interested in the latter, as they are undeniably implicated with the spread of respiratory infections like influenza. Droplets evaporate very quickly once they are released (in the order of milliseconds), and their size decreases to approximately half the original size of the droplet (Nicas *et al.*, 2005). Weber states that "there is no unique and generally agreed upon classification of airborne droplets, for example, concerning the aerodynamic diameter which defines the cut-off size between droplet nuclei and large droplets" (Weber and Stilianakis, 2008) (page 362). Droplet nuclei are generally considered to be smaller than 10 µm in aerodynamic diameter, although some studies cite 5 µm as the cut off.

It has been shown that viruses are more effectively spread by aerosol than bacteria or fungi (Carducci *et al.*, 1999). Barker and Jones (2005)reported that in a controlled study on aerosols generated from toilets, twice as many viruses survived than bacteria. Viral diseases thought to be transmitted by infectious aerosol range from relatively mild conditions such as the common cold to more severe diseases, including severe acute respiratory syndrome (SARS), smallpox and influenza (Tseng and Li, 2005, Wang *et al.*, 2005).

Several studies have successfully detected influenza virus in droplets created in a simulated, laboratory setting, with widely varying results (Hemmes *et al.*, 1960, Harper, 1961, Mitchell *et al.*, 1968, Mitchell and Guerin, 1972, Schaffer *et al.*, 1976, Ijaz *et al.*, 1987, Blachere *et al.*, 2007, Fabian *et al.*, 2009b, Sedlmaier *et al.*, 2009). This variance may be attributed to the fact that the studies were conducted over an extended time period, and employed widely varying methods (Nicas *et al.*, 2005). Only one available study was able to

detect influenza viruses in droplets expelled by infected individuals (Fabian *et al.*, 2008); none have quantitatively examined the distribution of influenza in droplets expelled by humans. However, two studies have reported quantitative data regarding the detection of influenza viruses in simulated droplets (Harper, 1961, Fabian *et al.*, 2009a).

Loudon and Roberts (1967) stated that the aerial transmission of infection involves three stages: production, transport and reception of droplets. This study focuses on the transport stage (and subsequent 'survival' of viruses), however we must first look at the production of droplets to understand more about the resulting bioaerosols and how the fate of the organisms will be affected.

1.2.2.1 Production of bioaerosols

The emission of respiratory droplets by people can occur via breathing, talking, coughing or sneezing (Edwards *et al.*, 2004). Droplets are created when currents of air pass over the mucous linings in the respiratory tract, creating disturbances in the surface of the mucous. This can lead to the creation of droplets which break away from the bulk of the liquid (Fiegel *et al.*, 2006). The production of droplets and aerosols is a complex process in the body, with many factors influencing the properties of the resulting particles.

Droplets expelled from the respiratory tract differ in size depending on the exact point of creation. This is due to the physiology of the respiratory tract: as the diameter of the respiratory tract varies, air pressure and speed change accordingly (Morawska, 2006). For example, the trachea has a larger diameter than bronchioles, so the air pressure and speed is lower, and the resulting droplets theoretically should be larger in size and fewer in number.

The properties of mucous and respiratory fluids will most probably introduce a further influential element in the creation of the resulting droplets. Edwards (1991) noted that high surface tension of mucous, which is found in infected individuals, favours the formation of relatively larger droplets; similarly, lower viscosity favours the formation of smaller droplets (Burkdolder and Berg, 1974). Differing compositions of respiratory fluids will require varying flow velocities in the respiratory tract to produce droplets, due to changed adhesion forces of the respiratory fluids (Papineni and Rosenthal, 1997). In an infected individual, these fluids will usually be more viscous, and therefore it can be inferred that droplets produced by such an individual will be different from that of a healthy individual. Therefore, droplets will vary in size, in the number of droplets produced, according to the type of respiratory activity and the health status of the individual.

Talking and breathing activities generally tend to create droplets which are larger and fewer in numbers. Droplets from these activities are typically created in the mouth. Papineni and Rosenthal (1997) found that there were many small particles in the exhaled breath of healthy subjects. Breath aerosol formation was recently attributed to the bursting of respiratory films or bubbles in the lower bronchioles, occurring during the initial stages of inhalation; this is known as the bronchiole fluid film burst (BFFB) model. It opposes the original assumption that breath aerosol is formed by the turbulence-induced mechanism. This research found that rapid inhalation and deep exhalation increased the aerosol concentration in subjects' breaths (Johnson and Morawska, 2009).

Respiratory activities such as coughing produce droplets which are much smaller, and in greater numbers, usually from the lower sections of the respiratory tract. Here, the respiratory passage is narrow and thus the wind speed is higher, so more pressure is exerted on the respiratory fluids, creating smaller droplets. Droplets from sneezing are more likely to come from the nasal passages whereas coughing produces droplets from the lower respiratory tract. Droplets produced from coughing and sneezing were reported to range in size from 1 to 100 µm in one study (Kowalski and Bahnfleth, 1998) and from 1 to 20 µm in another study (Knight, 1973). Papineni and Rosenthal (1997) reported that the quantity of droplets produced was highest in coughing compared to other respiratory activities.

The creation of the droplet does not only affect the droplet physically, in terms of its fate in the environment, but ultimately it dictates whether or not the droplet will contain an infectious particle. In this regard, we must consider the general pathologies of respiratory infections. The precise location of the infection in the respiratory tract is certainly the most important factor here. Some viruses, for instance rhinoviruses and other viruses causing the common cold, preferentially localise infection in the upper respiratory tract. Other viruses favour the lower respiratory tract. Parainfluenza viruses and respiratory syncytial viruses both replicate in the nasopharyngeal epithelium, and after a few days spread to the lower respiratory tract (Hall, 2001).

Avian influenza (influenza A subtype H5N1) has been found to localise in the lower respiratory tract of humans, making this a relatively severe form of influenza with a high mortality rate (World Health Organisation, 2010a). However, it did not spread efficiently amongst people, and consequently pandemic status was not conferred. Contrastingly, the more recently observed swine influenza (H1N1) spread very easily. Interestingly, swine flu, as well as most types of seasonal influenza, localises in the upper respiratory tract (Shinya

et al., 2006, van Riel *et al.*, 2010). The mortality rate of swine flu was far lower than that of HPAIV, even less than that of normal seasonal influenza, and the symptoms were much more mild.

The interaction between an influenza virus and the type of sialic acid group on the adjacent cells of the respiratory tract determines the binding of the virus (known as virus tropism), and thus the capacity for infection. Studies that identify specific binding receptors in the human respiratory tract, called sialic acids, which are ligands for influenza (Nicholls *et al.*, 2008), have been used to determine the tropism of HPAIV for the upper and lower respiratory tract. Generally, research has revealed that HPAIV preferentially bound to the receptors in the lower respiratory tract, but did demonstrate some binding in the upper respiratory tract.

A review of the data suggests that viruses that infect the upper respiratory tract generally are more easily spread, but less severe than those that infect the lower respiratory tract. This, presumably, is a result of the mode of production of bioaerosols, as opposed to the transport mechanisms of the viruses.

1.2.2.2 Transport of bioaerosols

An area of specific interest of the three stages of aerosol transmission is transport of droplets. Bioaerosol transport in the air has been widely documented in field or model studies. However, fewer studies have examined the aerial transport and fate of droplets emitted by humans during breathing, coughing, sneezing or talking (Nicas *et al.*, 2005). Even fewer studies have examined the detection of influenza virus in infected individuals; before 2007 there were no known studies that had achieved this.

Morawska (2006) stated that the most important factor affecting particle fate is particle size; additionally, particle size has a significant effect on the biological properties of a droplet. The initial size and concentration of the droplets alters dramatically upon release into the atmosphere, mostly due to evaporation. Droplets of pure liquid evaporate completely; thus, the degree by which the droplet evaporates is largely dependent on the water content and composition of the droplet. Interestingly, a recent study found that the dynamic evaporation of respiratory aerosols was very similar in speed to pure water droplets of a similar size; however this was only investigating dynamic evaporation in the order of seconds, and does not preclude further drying of droplets over minutes or hours (Morawska *et al.*, 2008). Furthermore, it investigated droplets expelled by healthy individuals. If during respiratory infection, droplets have a higher mucous content (and thus

less water content), the particle size may not decrease in size upon release, compared to a droplet with less mucous content (and more water content).

Once the droplets have been released into the air, they are subjected to a number of forces that determine their distance and time of travel; these include Brownian motion, gravity, electrical forces, thermal gradients, electromagnetic radiation, turbulent diffusion and inertial forces. (Pillai and Ricke, 2002). Relative humidity also plays an important role in droplet fate. Brownian motion, which refers to the collision of the droplets with other molecules in the air, increases with rising temperature and decreasing size; particles larger than 1 μm are generally more affected by gravity than Brownian motion (Pillai and Ricke, 2002) and may settle rapidly out of the air (Papineni and Rosenthal, 1997, Foarde *et al.*, 1999). Other factors affecting droplet fate are the size, shape and quantity of the droplets.

Information on droplets expelled by healthy individuals is relatively abundant. It has been reported that the size of droplets released by humans can range from 0.5 μm to 200 μm (Erdal and Esmen, 1995). Morawska and colleagues (2008) identified three distinct modes of aerosols during common expiratory respiratory activities: breath mode aerosol with a count median diameter (CMD) of <1 μm; a vocal cord vibration aerosol with a CMD near 3 μm; and a saliva aerosol mode near 10 μm. The number and concentration of particles is highly variable from person to person. Edwards and colleagues (2004) studied the release of droplets by 11 subjects and were able to separate them into two groups based on the number of particles they exhaled. Whilst 'high-producers' expelled an average of 500 particles per litre per six hours, 'low-producers' expelled an average of less than 500 particles per litre per six hours. However, it must be noted that the subjects in the preceding studies were healthy volunteers, and that the droplets produced from healthy individuals are likely to be dynamically distinct from the droplets that infected people produce.

Information on droplets expelled by infected individuals is vital to the understanding of transmission of infection, however, this issue has not been appropriately pursued. Additionally, publications describing particle size are not always clear as to whether droplet measurements refer to the initial droplet size (size before evaporation) or the dry droplet size (after evaporation) (Morawska *et al.*, 2009). This difference is important, as evaporation plays a very important part in droplet fate. It is imperative to know the final size and composition of the droplets in question, so that their fate can be accurately predicted.

The majority of reports published on bioaerosol transport have agreed on the most influential factors that affect virus transport in the air. The most commonly investigated environmental condition that has been documented is relative humidity (RH). It has been reported that some respiratory illnesses are less frequent in high RH (Wang *et al.*, 2005); however this is not the case for all viruses, as one study found that rhinoviruses had a higher recovery rate at high RH. Lipid-containing viruses tend to have a greater stability at lower RH, usually below 40 % (Benbough, 1971, Pillai and Ricke, 2002).

Until recently, little research had been conducted on the airborne characteristics of influenza. Most work conducted was done in a simulated environment. Several researchers have investigated the effect of RH on influenza. One found that the virus is rapidly inactivated at RH over 50 % (Hemmes *et al.*, 1960). A second publication reported that the best recovery rate of intact influenza was at a low RH and temperature (Sobsey and Meschke, 2003). Furthermore, high RH causes aerosol droplets to increase in volume, which results in higher fallout of the droplets (Stewart, 2002). In contrast, Gerone and colleagues (1971) concluded that RH had little or no effect on the recovery efficiency of viruses. The generally accepted view, however, is that low temperatures and low RH favour recovery of viruses in aerosols. This is one of the explanations as to why influenza epidemics occur during winter periods.

In an early example of a study on airborne influenza, Mitchell and co-workers (1968) investigated the aerosol characteristics of influenza A from human and avian origin, and found that influenza A of avian origin remained intact for longer periods in the air than those of human origin; avian influenza was therefore considered to pose a greater risk of infection. The same authors subsequently published a paper with the aim of examining strains of influenza A with origins in other species (Mitchell and Guerin, 1972). They found that influenza A of human and swine origin were more susceptible to decay than those of avian and equine origin.

A surge in funding for influenza research following the avian influenza scare is evident in the number of papers published in the last couple of years. Similarly, a number of review articles have been published, mostly consolidating data from the few sporadic papers that were published over the last eight decades or so, prior to the increased research in this area. These reviews have confirmed what was already known to researchers in this field: that little data exists; that the existing data is rather conflicting and highly variable; that aerosol transmission is potentially an extremely important mode of transmission; and that

more research is needed to confirm this. In addition, some reviews have raised some novel ideas.

One such review used the results of selected publications as evidence to support the use of a method for suppressing the natural expiration of droplets by infected individuals, in order to prevent widespread dissemination of respiratory infectious diseases (Fiegel *et al.*, 2006). From their survey of publications, the authors were able to conclude that both inhaled and exhaled bioaerosols could act as vectors for deep-lung and environmental transport of airborne disease. They also concluded that it is likely that a small percentage of the population would be responsible for dissemination of the majority of exhaled bioaerosols.

Tellier (2006) reviewed the evidence for aerosol transmission of influenza A, stating that published results from papers spanning the last seven decades highlighted the importance of aerosol transmission of influenza A as a mode of transmission. Some studies reviewed in this paper supported the theory that the preferred site of influenza virus infection is the lower respiratory tract. This was demonstrated by prevention of influenza transmission in individuals treated with zanamivir (a prophylactic neuraminidase inhibitor), where droplets of the drug were inhaled into the lungs. Contrastingly, intranasal application of the drug did not protect individuals from acquiring the disease. A previous study had demonstrated the efficacy of zanamivir in prevention of a laboratory-acquired influenza infection in humans (via intranasal challenge). Tellier (2006) concluded that this demonstrated that the virus was transmitted via the airborne route to the lower respiratory tract, and that aerosolisation is a viable natural route of infection. This finding shows that there is a need for a greater understanding of the types of droplets released by infected individuals.

Tang and co-workers (2006) conducted a review into aerosol transmission of infectious diseases within healthcare premises. From the data they gathered, the authors found evidence that infectious organisms are able to be transmitted over both short and long distances, and that some organisms associated only with short-range transmission can also cause outbreaks over greater distances via transmission of evaporated droplets. The review concluded that personal protective equipment is needed to prevent person-to-person short-range transmission of infectious diseases.

More recent studies investigating the aerosol transmission of viruses have elected to use bacteriophage in place of the specific virus; this is most likely because phage has a highly reduced health risk, as it is non-pathogenic. Bacteriophages are very useful in this manner, but may not always be entirely indicative of the virus they are representing. However, they

present an alternative method of investigation that is easy to analyse and process, and is safe to use for large scale studies.

1.2.2.3 Bioaerosol reception

The reception of droplets and subsequent risk of infection relies on the deposition of droplets in the respiratory tract, which in turn depends on a number of factors. The size of the droplet will determine the depth to which the droplet is able to penetrate the airways. A study conducted by Hatch (1961) found that the retention of particles in the respiratory tract is close to 100 % for particles with a diameter of about 5μm; retention drops to about 20 % for particles of about 0.25 -0.5 μm and then increases again to reach 60 % for sub-microscopic particles (smaller than 0.1 μm). The majority of droplets that are 20 μm in diameter deposit in the nasal passages, with a small fraction depositing in the pharynx and larger bronchi (Knight, 1980). Foarde (1999) stated that nearly all 2 μm particles are deposited in the respiratory tract, whilst Daigle and colleagues (2003) found that deposition of ultrafine particles (particles less than 0.1 μm in diameter) increased as their size decreased.

The smaller the particles, the more likely they are to impact on the lower respiratory tract. Proctor (1966) stated that although few deposition studies have been conducted, it is likely that particles smaller than 5 μm will penetrate to the lower respiratory tract. It has been reported that the main mechanism for deposition of particles with a diameter less than 0.5 μm is diffusion (Daigle *et al.*, 2003), which can occur in the alveoli of the lungs (Wilson *et al.*, 2002). Diffusion by smaller particles is more harmful because they can diffuse rapidly through the alveolar membrane into the blood (Hogan *et al.*, 2005).

Another factor in bioaerosol reception is the rate of inspiration, expiration and tidal volume of the individual (Heyder and Rudolf, 1977). The faster the airflow rates, the more particles deposit in the nose, and this increases with increasing particle size (Knight, 1980).

Hinds (1982) described the fate of aerosol particles deposited in the respiratory tract as such: the upper respiratory tract has a mucous lining that is propelled, along with the deposited particles, up toward the pharynx and swallowed; whereas the lower respiratory tract, including the alveolar regions, has no mucous lining, and clearance of particles is much slower and less efficient (Hinds, 1982).

Once a bioaerosol has deposited in the respiratory tract, whether or not a disease state will eventuate is determined by the following: whether or not the virus in question is still intact

and able to infect a cell; the location of deposition in the respiratory tract, and whether or not the virus can recognise the sialic acids on the cells, to which influenza virus binds (dependant on the neuraminidase); and the immunological status of the individual.

1.2.2.4 Bioaerosols in indoor environments

It is thought that the airborne spread of disease is more likely to occur indoors, because in outdoor environments, aerosols are rapidly dispersed (Knight, 1980). The transmission of viral illnesses in indoor environments is a matter of growing relevance to our society, with an increase in the use of air conditioners and poor ventilation in large buildings, often crowded with people. Contamination of indoor air has been related to 'sick building syndrome' and building-related illnesses. Considering that people spend 80-90 % of their time indoors in air conditioning, this could be a major issue (Scarpino *et al.*, 1998). This is particularly likely in shopping centres and buildings in business districts, where many people can occupy a small area in a short space of time. If a host suffering from a highly transmissible strain of influenza or other infectious disease was present, the spread of that illness in such an environment would be difficult to impede; this has been clearly demonstrated by the outbreak of severe acute respiratory syndrome (SARS), particularly evidenced in the Amoy Gardens apartment complex (Yu *et al.*, 2004, Li *et al.*, 2005b).

Large inner city buildings are not the only type of buildings that are effective in facilitating spread of infections. One study reported that respiratory illnesses were more common in army recruits who were housed in poorly ventilated, modern barracks than in recruits who stayed in barracks that were naturally ventilated (Brundage *et al.*, 1988). According to Fisk (2000), the theoretical relationship between the indoor building environment and the transmission of illnesses depends on the mechanisms of transmission. Incorporation of outside air into buildings with improved air ventilation was recommended to reduce the spread of infectious disease in indoor environments.

A study which successfully applied a theoretical model to a small scale chamber and a full-scale environmental chamber (simulating a room in an office) described the fate of particles in indoor environments (Gao, 2007). Looking at three different ventilation systems, it found that submicrometre particles were similar to tracer gases, in that they were not as affected by ventilation type as their larger counterparts, and tended to follow the mainstream air flow. Particles larger than 2.5 µm were more susceptible to gravitational forces, and this effect increased proportionally to particle size. Furthermore, human exposure to the particles increased as particle size decreased. For particles with a

size of 20 µm and above, the gravitational forces are so strong that most of these particles will fall out of the air and not be inhalable.

The spread of particles, and in particular bioaerosols, in indoor environments is a complex process, involving many factors. To understand more comprehensively the potential spread of disease in these situations, we require a more thorough understanding of virus persistence in droplets and the physical factors which significantly affect this.

1.2.3 Bacteriophage applications

1.2.3.1 Bacteriophage Structure and Function

Bacteriophages are viruses that infect bacteria, and are thus are not harmful to humans. Many bacteriophages exist, and are classified based on their morphology. Like other viruses, the genome can consist of either single- or double-stranded DNA or single- or double-stranded RNA (Nelson, 2004). In general, bacteriophages are smaller than the viruses that infect plants or humans, and their size and shape vary widely. MS2 is a non-enveloped, single-stranded RNA bacteriophage that infects *Escherichia coli*. It is male-specific, spherical, has a genome size of 3,569 bp (with four genes) and is easily propagated in the laboratory (Barker and Jones, 2005). It is a member of the Leviviridae family and the genus Levivirus, serogroup I (Bollback and Huelsenbeck, 2001). It does not naturally occur in the environment, and so use of this phage is useful for field studies, as contamination is not an issue.

1.2.3.2 Application of bacteriophage as a model

Bacteriophages have played an important part in various types of research. One such area in which their use has been of great significance is in molecular biology, where they have been used to study replication, translation and the role of secondary structure in gene regulation (Bollback and Huelsenbeck, 2001). Another important application of bacteriophages is their use as models or surrogates. In these systems, they are used in place of other viruses to give an indication of how the virus in question may respond to particular conditions. This use of bacteriophage is important because they do not pose serious health risks and are relatively easy to propagate in the laboratory; therefore there are reduced risks and costs associated with the experiments. They are also readily available and have relatively simple detection assays (Van Cuyk *et al.*, 2004).

Surrogate bacteriophages are selected based on a simple set of general criteria. The chosen bacteriophage is usually of similar size and shape of the virus, and exhibits similar

properties to that of the virus. For example, in an aerosol study, it is important to choose a bacteriophage that has similar aerosol characteristics to the specific viruses (in terms of aerodynamics and recovery). MS2 is a commonly used bacteriophage in surrogate studies, and it has been used as a surrogate in a variety of studies, such as examination of soil treatment of wastewater (Van Cuyk *et al.*, 2004), and dynamic batch experiments for groundwater, soil and air (Thompson and Yates, 1999). It is also reported to have similar aerosol characteristics as human viruses (Foarde *et al.*, 1999).

MS2 has also been used as a surrogate for viruses in aerosol studies, including some studies investigating influenza transmission in the air. Foarde and co-workers (1999) chose to use MS2 as a surrogate for various viruses including influenza, as it had similar shape and aerosol characteristics as human viruses, despite the fact that it is slightly smaller and non-enveloped. In another study, the authors chose MS2 as a surrogate for viruses such as smallpox and Ebola to study their fate in the indoor environment (Utrup and Frey, 2004).

In a third study, MS2 was used along with another *E. coli* phage, T3, to determine the best method for studying airborne viruses (Hogan *et al.*, 2005). It was chosen as a surrogate as it had suitable size and shape, similar to that of commonly tested airborne viruses. Barker and Jones (2005) used MS2 to examine aerosol contamination caused by toilets. It was favoured for this study because it is known to be relatively stable in the environment. MS2 was also used to test the emission rates and plume characteristics of bioaerosols generated from land application of liquid Class B solids (i.e. fertilisers derived from municipal sewage sludge) (Tanner *et al.*, 2005). Furthermore, it has been used to test an air purification system (Griffiths *et al.*, 2005), and to compare the efficiencies of common air samplers (Tseng and Li, 2005).

Bacteriophages infect bacteria, so they are much easier to collect and culture in the lab than viruses that infect animal and human hosts. MS2 can be collected onto an agar plate with an *E. coli* lawn, and the overlay method is used to clarify plaque formation (O'Connell *et al.*, 2006). Plaques, which appear as holes in the bacterial lawn, are counted, giving a plaque-forming unit (PFU) per plate or per litre of air collected, depending on the collection method. Additionally, MS2 like other viruses can be detected using PCR. A published paper by O'Connell and colleagues (2006)outlined five different assays for the detection of MS2 using a real-time, reverse transcriptase (RT)-PCR method, with TaqMan chemistry.

1.2.4 Aerosol methodology

Aerosol studies all have three common features: a source of aerosolisation, a method of air collection, and a method for detection of the desired organism(s). These features will be described in detail in the following sections.

1.2.4.1 Methods used to generate aerosols

Whilst some field studies measure the natural aerosolisation of a particular source e.g. aerosols from a sewage plant or from a toilet, others simulate aerosolisation using a nebuliser, which is a piece of equipment that can produce droplets (often of a uniform size). To use a nebuliser, information regarding the typical size and concentration of the natural process being simulated is required, in order to give the most valid results. The most commonly used nebuliser is the Collison, which was first described in 1935 by Collison (May, 1973). The Collison nebuliser uses compressed air (or gas) to aspirate a suspension into a gas jet, where it is sheared into droplets. The larger fraction of the droplets is then removed through impaction of the liquid/gas jet against the side of the jar, and the remaining droplets are passed out of a nozzle and into the atmosphere. The droplet size is modified by using different air pressures. Collison nebulisers have been employed in many studies (Gerone *et al.*, 1971, Ijaz *et al.*, 1985, Scarpino *et al.*, 1998). They are available in several versions, the most common being the six-jet Collison. A study that compared Collisons with a plastic nebuliser and a glass nebuliser found that the Collison, unlike the other types, showed very little variation and gave reproducible results. The authors also described the nebuliser as giving a constant spray volume when the fluid level in the reservoir was varied (Hino and Sato, 1984).

A similar version is the three-jet Collison. The main difference is that it has half the volume capacity of the six-jet; this would probably not be too detrimental in a study involving a small chamber as only small amounts of atomised solution are required. The three-jet Collison has been used in studies to generate aerosols containing bacteria and rhinoviruses (Scarpino *et al.*, 1998), and aerosols containing MS2 (Tseng and Li, 2005).

A problem associated with nebulisers is frothing of certain solutions. This can be corrected with the addition of an anti-foam solution. For example, antifoam A was added into the atomising solution containing virus and tryptose phosphate broth (TPB) (Karim *et al.*, 1985, Ijaz *et al.*, 1987). Other suspensions used for aerosolisation include phosphate buffered saline (PBS) (Myatt *et al.*, 2003), potassium chloride (KCl) (Foarde *et al.*, 1999) or deionised water (Tseng and Li, 2005). The volume of solution in the reservoir is typically 10-20 mL.

Another problem encountered with Collison nebulisers, which has been raised by several researchers, is that the process of nebulisation causes loss of viability to the microorganisms. This is not necessarily always the case however, and Ijaz and colleagues (1987) found that the Collison did not cause injury to poliovirus or rotavirus.

1.2.4.2 Methods used to collect droplets

Early in the 20th century, before mechanical air samplers were in common use, studies investigating airborne organisms used agar settle plates for collection. However, this approach is no longer widely used as it has been replaced with more efficient and informative means of sampling. Indeed, there are some serious disadvantages of the settle plate method, including that the plates cannot be left out for long periods in case the agar dries out; the volume of air cannot be measured; and not all particles will settle onto the plates. However, in suitable conditions, this sampling method may still be valid, despite its primitive nature, for some studies. Such conditions should be controlled to minimise the amount of experimental variables, and may include a smaller sampling volume (i.e. a small chamber would be more suitable than an entire room), with the aerosolised organisms generated in droplets of known concentration and size. If suitable conditions are met, then this method would have the potential to provide valid information; specifically, a general spatial distribution of the droplets would be obtained. This method could potentially be more useful if coupled with a second method, for example an aerodynamic particle sizer (APS) to measure the size distribution of the droplets, or an air sampler to measure the volume of air.

A wide range of air sampling devices exist (Eduard and Heederik, 1998), with some used more universally than others. Air samplers collect air by various techniques including liquid impingement, solid agar impaction and gelatine impaction. Two samplers that use impingement into fluid are the All Glass Impinger (AGI-30) (Ace Glass Inc., Vineland, US) and the SKC BioSampler® (SKC, Pennsylvania, US). Samplers can also collect by impaction onto a solid (or semi-solid) surface, such as the Andersen microbial impactor. Each type of sampler has both advantages and disadvantages with respect to their functionality and adverse effects on the aerosols. One disadvantage of the impinger samplers is that during sampling the collection medium evaporates, which can potentially lead to loss of the sample. This is particularly problematic if collecting large volumes of air: the longer the samplers operate, the more evaporation occurs. The SKC BioSampler® can be used with a non-evaporative medium, which has been found to be suitable for long-term sampling of

bacteria and fungi (Lin *et al.*, 1999). It has also been used for sampling of viruses (Agranovski, 2007). Another disadvantage of impingers is that the flow rate of the vacuum used to sample the air is quite low, and thus sampling can be lengthy. The advantage of collecting organisms into a liquid is the flexibility afforded in terms of sample processing. The samples can be used to inoculate a growth medium, or can be used for molecular analysis (i.e. PCR).

A recent study examined sampling parameters to optimise a sampling system using an impinger for the collection of airborne porcine reproductive and respiratory syndrome virus (PRRSV) and swine influenza virus (SIV) (Hermann *et al.*, 2006). This study examined specific aspects of this air sampler, including media compound additives which are often incorporated into the collection media to enhance collection and recovery of the droplets. For example, antifoam is usually added to the impingement fluid, as this method results in excessive foaming. The effects of such compounds on the specific aspects of virus detection had not been previously explored.

The study found that using RT-PCR, the detection of both PRRSV and SIV was not affected by antifoams or ethylene glycol, but was adversely affected by the addition of protectants and sorbents (activated carbon products). The suggested explanation for this was that the activated carbons inhibited the PCR, or that the virus was adsorbed onto the carbon and was thus unavailable for the PCR assay. In addition, ethylene glycol proved cytotoxic to the continuous cell line on which the virus was grown. The authors emphasised the fact that the effect of each compound needed to be examined in the context of its intended use.

The same study also examined sampling parameters focussing on the functionality of the equipment. They reported that the SKC BioSampler® and the AGI-30 models recovered more virus than the AGI-4 model. They also found that phosphate buffered saline (PBS) with ethylene glycol used as the collection medium gave a greater recovery of the aerosolised PRRSV, but again stated that each factor should be examined within its intended context (Hermann *et al.*, 2006). The authors concluded by noting that there is currently no standard method for the detection of specific pathogens in aerosols, and that this is a largely under-explored area of research.

The Andersen six-stage impactor sampler is a cascade impactor, which is capable of obtaining information on the size distribution of aerosols containing organisms. Bioaerosols are deposited onto agar plates in each of the six stages, separating out according to the size of the particles (see figure 1-3). Each stage has 400 impaction holes, the size of which

decrease from stage one to stage six, with the largest particles collected in the first stage and the smallest collected in the last stage (Griffiths and Stewart, 1999). Stages one to six have 50 % cut-off diameters of 7, 4.7, 3.3, 2.1, 1.1 and 0.65 μm respectively. So stage one collects particles 7 μm and larger, stage two collects particles 4.7 μm to 7 μm, and so on (Russell *et al.*, 2002).

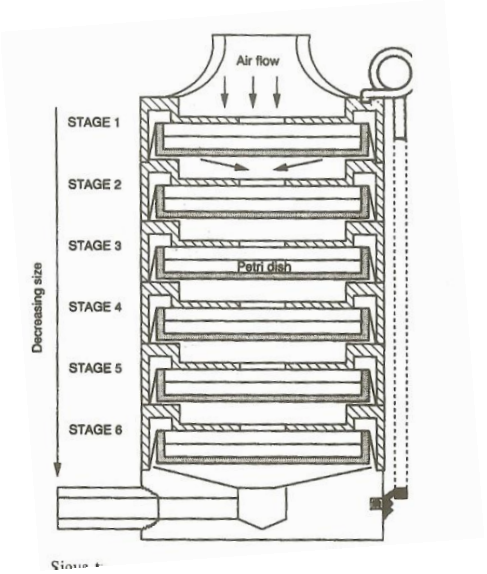


Figure 1-3 Schematic diagram of the six-stage Andersen impactor

The designed flow rate of the six-stage sampler is 28.3 L/min (Eduard and Heederik, 1998) and is regulated by a vacuum pump which draws the air into the sampler. Sampling errors of particles using Andersen samplers have been reported, but in indoor conditions, aspiration losses of particles smaller than 10 μm are small (Grinshpun *et al.*, 1994). Upton and colleagues (1994) found that the physical collection efficiency of the Andersen was greater than 90 % for particles smaller than 2.5 μm, and at low wind speeds, the collection efficiency was 100 % for particles as large as 20 μm. Sample processing can be shorter than other samplers, especially if the organism being collected can be collected directly on the culture medium.

Several comparative studies have been conducted to determine which of these samplers has the best collection efficiency. However, most of these studies have agreed that the choice of air sampler depends on the nature of the experiment. One study compared some common air samplers for collection of bacterial cells and spores. Results showed that using the Andersen impactor, bacterial cells had a higher recovery rate than when an RCS

centrifugal impactor was used at both 30% and 70% RH. The Andersen also yielded a higher recovery of the bacteria than the Parrett MicroBio sampler at 30% RH, although the rate was similar at 70% RH (Griffiths and Stewart, 1999). Tseng and Li (2005) compared the collection efficiencies of four different samplers using MS2: a one-stage Andersen, an AGI-30, a gelatin filter and a nucleopore filter. For this study they used a six-stage Andersen as a reference sampler to measure the size distributions of the aerosols. The authors found that when using a one-stage Andersen, RH did not affect the relative recovery of phage. They concluded that the Andersen one-stage impactor, the AGI-30 and the gelatin filter sampler were more suitable than the nucleopore filter for collection of aerosols containing viruses.

The Andersen impactor, in particular the six-stage cascade sampler, has been recommended as a reference sampler (Eduard and Heederik, 1998, Foarde *et al.*, 1999). It is the sampler of choice when measuring the size distribution of aerosols, as it is able to separate the particles into size fractions onto the agar plates. Moreover, the use of a stacked sieve sampler, like the Andersen, has been recommended in experiments where the concentration of the organisms is expected to be low (Brachman *et al.*, 1964).

There are some problems that have been encountered with the Andersen sampler. One issue is that impaction onto the agar surface can cause injury and loss of viability to the organisms. This injury may result in an underestimate of the number of organisms collected. Nonviable organisms cannot be counted on the plates, as they are uncultivable; they can only be detected using PCR (Foarde *et al.*, 1999). The loss of viability is not easy to assess and is not always constant (Brachman *et al.*, 1964). Grinshpun and co-workers (1996) proposed that by using an impactor with a moving surface, less injury might be incurred on the organisms. However, whether or not impaction results in loss of virus infectivity has been questioned. It was reported that RNA was not degraded during the sampling process with the Andersen; rather, some of the RNA was lost during the alcohol precipitation process in the RNA extraction (for PCR) (Russell *et al.*, 2002).

Further, Kenny and colleagues (1999) stated that although the Andersen sampler allows separation of particles based on their size, the fractions aren't in accordance with the current ISO-CEN definitions for health-related sampling (divided into inhalable, thoracic and respirable fractions). Another issue when using an Andersen impactor is that the organisms are generally not collected into a liquid medium (as in the AGI-30), but rather a solid or semi-solid medium. The advantage of liquid collection media is that it can be analysed directly (PCR or other), whereas to do this with an Andersen, the organism must be washed from the agar surface using a buffer; some of the organisms may be lost in this

process. On the other hand, the use of liquid media when long sampling periods are needed results in loss of the collection medium containing the organisms by evaporation.

It is difficult to collect viruses on agar surfaces because there is no standard collection medium or protocol for this application. However, the Andersen sampler has previously been used to collect airborne viruses: Ijaz and co-workers (1987) used the Andersen and modified an agar used by Guerin and Mitchell (1964) for virus collection. A gelatin solution was added into TPB to a final concentration of 3%. It was also used to collect human rhinovirus from infected volunteers for PCR analysis, although no rhinovirus was detected on any of the stages (Russell *et al.*, 2002). This could be attributed to: the low number of volunteers who were confirmed to be infected with the rhinovirus; the use of the six-stage sampler, which may have spread the distribution of any viruses collected over the six stages; the volume of liquid in which the samples were collected (diluting the samples); or the actual method of virus collection, which was conceded by the author.

Another common problem with impactor type samplers is the so called overloading of the sample plates. If used to sample from environments with high concentrations, more than one viable biological particle may deposit on the same spot on the plate. If culture methods are used these particles will be counted as one resulting in an underestimation of the actual concentration. A paper published in 1989 described this, and produced a table that could be used to correct the microbial figures collected, based on those which were actually counted after collection, and the probability of this occurring (Macher, 1989). This process is called ‘positive hole correction’.

New samplers have been devised in recent years to tailor the sampling process to the collection of bioaerosols, particularly to virus collection. There is particular interest in developing samplers which offer portability; ease of operation; simplified sample processing; and greater recovery of bioaerosols through collection methods which are less harmful to the organisms than traditional samplers. The drawback to these samplers is that currently, they are not commercially available.

One example of such a sampler was a new personal bioaerosol sampler (Agranovski *et al.*, 2006), usedin a study of airborne viruses, which had been previously developed and described (Agranovski *et al.*, 2005). In the study, the performance of the sampler was evaluated for collection of various bacteria and for influenza and was found to be a suitable instrument. Agranovski and co-workers (2006) described the combination of the sampler with PCR for more rapid detection of the selected organism. The study was carried out

using Vaccinia virus as a test organism. The virus recovery was then evaluated for both plaque assay and for the conventional PCR assay.

Another recently developed sampler is a cyclone-based, two-stage personal bioaerosol sampler, described by Lindsley and colleagues (2006). It offers a simplified process of sampling, whereby microcentrifuge tubes are used to collect the aerosols, and samples can be processed with PCR assays or immunoassays. It also has the added benefit of size fractionation of the collected aerosols. The size cut-offs can be altered according to the flow rate at which the sampler is operated. When operated at 2 L/min, the cut-offs of the two stages are 2.6µm and 1.6µm; when operated at 3.5 L/min, the cut-offs are 1.8µm and 1.0µm. This two-stage cyclone sampler has been used successfully to collect influenza viruses, which were analysed by PCR assay.

Vaccinia was chosen as the test organism because it is a robust DNA virus with a high recovery rate, and thus infectious and total virus concentrations should theoretically be very similar, which was demonstrated in this study (Agranovski *et al.*, 2006). Results of the study showed that the PCR was successfully integrated with the bioaerosol sampler, with high recovery rates of aerosolised virus ranging from 73-89%. However, when used with more sensitive viruses such as influenza and at lower concentrations, this method may not be sensitive enough to detect low concentrations of virus. The use of a more sensitive PCR, for example real-time PCR, would most likely be needed, as suggested by the authors.

Various studies have found that virtually all of the commonly used air samplers have disadvantages as well as advantages, and some authors have proposed means to rectify any errors in sampling, either in the sampling conditions, the operation of a sampler or in the analysis of the results. It has been stated that a representative sample of an aerosol can be collected if done so in isokinetic conditions (i.e. where the velocity and direction of air currents outside the air sampler are the same as those inside the sampler), and if this is not the case then the sampler may give an over- or underestimate of particles larger than 5-10 µm (Vincent, 1989). Ultimately, the choice of sampler depends on the type of experiment one wishes to conduct, and the advantages and disadvantages should be considered when making this decision.

1.2.5 Molecular methods in virology

In recent years, polymerase chain reaction (PCR) techniques have replaced cell culture methods as the gold standard assay for detection of viruses. In virology, PCR is used as a

molecular diagnostic assay to detect nucleic acids of the desired target organism. This is achieved with the use of a pair of synthetic primers (forward and reverse), which hybridise to a strand of double-stranded DNA (dsDNA). The region spanned by the primers is then multiplied exponentially, resulting in many copies of the nucleic acid sequence of interest. PCR is generally more rapid, sensitive and reproducible than culture techniques (Mackay *et al.*, 2002). Culture techniques require that the organisms are culturable, whereas PCR can theoretically detect nucleic acids of all of the microorganisms present in a specimen (Zhang and Evans, 1991). This is significant, considering that the culturable microorganisms are only a fraction of the viable microorganisms in samples (Eduard and Heederik, 1998). In addition, cell culture techniques often fail to detect virus in the respiratory secretions of patients in the latter stages of their illness (Cherian *et al.*, 1994).

Comparison of plaque assay and PCR assays is not straightforward, as they measure two totally different things: culturable organisms and nucleic acids, which cannot be correlated by any means we currently possess. However, there have been several publications which have sought to compare the efficiencies of each of these methods, by simultaneous application of both to samples (Atmar *et al.*, 1996, Rose *et al.*, 1997, Magnard *et al.*, 1999, Liolios *et al.*, 2001, Bae *et al.*, 2003, Perkins *et al.*, 2005). Some of these studies compared the assays using simple presence/absence data, and some went as far as to compare the quantification methods attached to the assays.

An early study, published in 1996, compared the use of a previously developed PCR assay with a viral plaque assay and a commercial rapid diagnostic test to detect influenza A in swab specimens (Atmar *et al.*, 1996). Only positive and negative results were recorded, with no comparative quantification attempted. The authors reported that the reverse-transcriptase (RT)-PCR gave very similar results to that of the viral culture assay, with a couple of negative results from assay-positive samples.

Rose and colleagues (1997) also produced one of the earlier publications that undertook a comparison of the two techniques. They compared plaque assay and reverse-transcriptase (RT)-PCR on marine samples, in the detection of coliphages. The authors used a most probable numbers method in combination with the PCR assay (MPN-PCR), and compared the results with those of the culture assay. They reported that the PCR assay gave positive results in samples where the plaque assay had returned negative results. However, they also found that the opposite was true: the plaque assay was able to detect coliphages where the PCR assay could not. Furthermore, the quantities estimated from each method did not have a high correlation.

More recent studies have been able to employ real-time PCR techniques, which have developed rapidly over the last decade. This method, which is described in detail later in this section, has the ability to quantify the number of genomes during the course of the assay (hence the name 'real-time'). Using this method, it has been possible to more accurately compare the quantification of viruses by culture methods and PCR methods. One example used both methods to determine the viral load of clinical samples containing RSV (Perkins *et al.*, 2005). They cited a ratio of 1 PFU to 153 genome copies. However, they too reported that the correlation between the results from the plaque assay and the PCR assay was low, demonstrating that the relationship between the two methods is not clear-cut.

Some other studies have cited ratios of $TCID_{50}$ values to virus particles, for example Schweiger and co-authors (2000) cite a particle ratio of 1:100, where 1 $TCID_{50}$ is equivalent to 100 virus particles (as measured by PCR). They did not, however, justify this ratio nor did they cite any reference that could do so. It is in fact not currently known exactly how many virus particles it takes to produce infection, or in the case of infectious dose methods, cytopathic effects. Furthermore, infectious dose methods and plaque methods do not correspond to one another, due to the fundamental difference in their assay principles. The $TCID_{50}$ method is expressed as the dilution of a virus culture at which cytopathic effects are seen in 50 % of cultures, whilst the plaque assay method measures how many plaques form when the virus is seeded onto a tissue culture.

Many assays have been developed for the detection of several respiratory viruses including influenza, parainfluenza (PIV) and human respiratory syncytial virus (RSV). Each of these viruses is a single-stranded RNA virus. As primers hybridise to dsDNA, the genomes of RNA viruses must first be converted to DNA. This is done by reverse transcription PCR (RT-PCR), where RNA is transcribed into cDNA using a viral reverse transcriptase. Researchers have focused in particular on developing assays for the detection of various strains (or subtypes) of influenza A, as it is an important pathogen, and common in respiratory samples. The first published PCR detection assays for influenza employed primers targeting the highly conserved genetic sequences of both influenza A and B. Zhang and Evans (1991) described the genome of influenza as genetically unstable, due to the constant mutation and evolution of the virus. Therefore, they designed two sets of primers which targeted a highly conserved gene, the matrix gene on segment seven of the virus genome. The assay developed was highly specific and sensitive, and made detection of the viruses simple. The

methods and primers designed have been cited in recent publications (Liolios *et al.*, 2001, Hindiyeh *et al.*, 2005).

Donofrio and colleagues (1992) designed primers, also targeting the matrix gene, which produced amplicons of 212 bp for influenza A and 365 bp for influenza B. The assay was shown to be a powerful means of detecting the virus and appropriate for use in epidemiological and diagnostic work. These primers are still in use and have been cited in recent studies (Atmar *et al.*, 1996, Ellis and Zambon, 2001).

A major advance in PCR techniques was the development of real-time PCR. This allows monitoring of the amplification of the amplicon and its subsequent quantification. This is made possible by labelling the primers, probe or the amplicon with fluorogenic molecules. There are five main methods for this: DNA binding fluorophores; the 5' endonuclease; adjacent linear oligoprobes; hairpin oligoprobes; and the self-fluorescing amplicons (Mackay *et al.*, 2002). Real-time PCR has also allowed an increase in the rapidity of the assay due to reduced cycle times, removal of post-PCR detection, labelling, and sensitive methods of detecting their signal (Mackay *et al.*, 2002).

A commonly used chemistry in real-time PCR is TaqMan® probe method, which was first reported in 1991 (Holland *et al.*, 1991), and then developed by Roche Molecular Diagnostics and Applied Biosystems. They rely on the measurement of fluorescence released where the probe is degraded by 5'-3' exonuclease activity of the Taq polymerase (Heid *et al.*, 1996). One PCR assay utilised TaqMan® technology to detect and subtype influenza viruses in respiratory samples. It used fluorogenic probes that were labelled with a 5' reporter dye, 6-carboxy-fluorescin (FAM) and the quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA). The probe was annealed to the target sequence (the amplicon) and upon cleavage, and the dyes were separated, resulting in increased fluorescence of the reporter (Schweiger *et al.*, 2000). TaqMan® technologies have been used in several other studies (van Elden *et al.*, 2001, Stram *et al.*, 2004, Hindiyeh *et al.*, 2005).

Real-time PCR assays allow quantification of DNA or RNA by use of a standard curve. Standard curves are a series of dilutions (of the target sequence) which are in known concentrations. A standard curve is constructed after amplification of a known quantity of the target sequence enters the log phase, and unknown samples can be quantified against the curve (Mackay *et al.*, 2002). This is not always accurate, however, as the standards, if prepared from cultures or stocks, can contain extraneous nucleic acids which may be amplified by the PCR assay, and thus give a false indication of the true amount of DNA or

RNA copies from an organism, if that is the intended application. Some studies have described methods whereby synthetic DNA or RNA is used in place of culture material, to reduce this problem (Collins *et al.*, 1995, Lefeuvre *et al.*, 2006, Smith *et al.*, 2006).

The techniques detailed above can be combined to develop an assay which is suited to the application of a study. Optimisation of methods is often required, as the objectives of different studies vary. For example, in one study, Stram and colleagues developed a multiplex, real-time RT-PCR using TaqMan® chemistry, to detect two viruses in clinical samples (Stram *et al.*, 2004). A second study developed an assay for the detection of airborne virus, with an increased sensitivity. This method was shown to be the only method available to detect low concentrations of respiratory virus in the air (Myatt *et al.*, 2003).

Several recently published articles, as reviewed by Peccia and Hernandez (2006), have reported PCR assays for the detection of microorganisms in aerosols. However, few actually demonstrated the application of PCR to aerosol testing to detect viruses. Some papers have reported successful application of PCR assays to viruses in aerosols, in particular influenza and other respiratory viruses (Hermann *et al.*, 2006, Blachere *et al.*, 2007, Fabian *et al.*, 2008, Fabian *et al.*, 2009b, Gendron *et al.*, 2010). Whilst application of qPCR has been successfully achieved by many laboratories, it is the quantification aspect of the studies that has not yet been perfected, and inconsistencies are evident in the results of these papers. This is not surprising, as a number of errors can be introduced during the multiple stages of sampling and sample processing, and when coupled with the exponential amplification of the collected nucleic acids in PCR assays, it is likely that such results may occur. This will be one of the major obstacles to overcome in the development of this application.

It is difficult to know what to expect in terms of RNA recovery in aerosols, because most studies do not report on the relative recovery of organisms in aerosols. Relative recovery is different to quantification because it is related back to the original concentration of viruses that were aerosolised. One study (Fabian *et al.*, 2008) reported the detection of up to 300 influenza virus RNA copies from human exhaled breath, however the quantification of influenza virus RNA copies from nasal swabs was not reported, thus the relative recovery cannot be estimated from these results. Another study, however, did report the relative recovery of bacteriophages MS2 and phi6 (Gendron *et al.*, 2010) at approximately 10^{-1} of the original suspension, as measured by quantitative PCR.

In a review by Peccia and Hernandez (2006), the authors attributed the general lack of information regarding the aerial transport of microorganisms to the deficiency of suitable detection systems, and poor integration of biotechnology with aerosol science. With advancing efficiencies in aerosol sampling and sample processing and the development of increasingly sensitive and accurate qPCR assays, their combined application will greatly advance our knowledge of the transport of viruses in respiratory droplets.

1.3 Overall aims of the study

The hypothesis of this thesis was 'that RNA viruses may be detected in aerosols using a purpose-designed assay system that is sufficiently sensitive'. The overall aim of this project was to examine the distribution of viruses in respiratory droplets in simulated indoor environments, and to determine the effect of various physical factors on virus integrity, such as length of time that viruses remain intact in aerosols, and the concentration of the virus in the droplets. This will be achieved with the following three aims.

1.3.1 Development of PCR assay

The first aim was to develop a sensitive PCR assay to detect and quantify potentially stressed or damaged viruses in droplets. PCR assays were developed for bacteriophage MS2 as well as for four respiratory viruses: influenza A (H1N1 and H3N2 subtypes); influenza B; parainfluenza virus (subtype 1); and human respiratory syncytial virus.

1.3.2 Bacteriophage experiments

The second aim was to examine the behaviour and recovery of aerosolised bacteriophage within a sealed chamber, including individual droplet size, limiting physical factors, and the relative recovery of viruses in various conditions. The PCR technique developed in the first objective was applied to samples collected specifically for this purpose, and results were reviewed in parallel with the traditional culture techniques. Both liquid and mucous-like suspensions were compared to determine if they are significantly different, and to obtain a closer simulation of real-life situations. Bacteriophage MS2 was selected as the surrogate for respiratory viruses due to its similar size and shape, and prior use in similar studies. MS2 was expected to behave in a similar manner to the respiratory viruses.

1.3.3 Influenza experiments

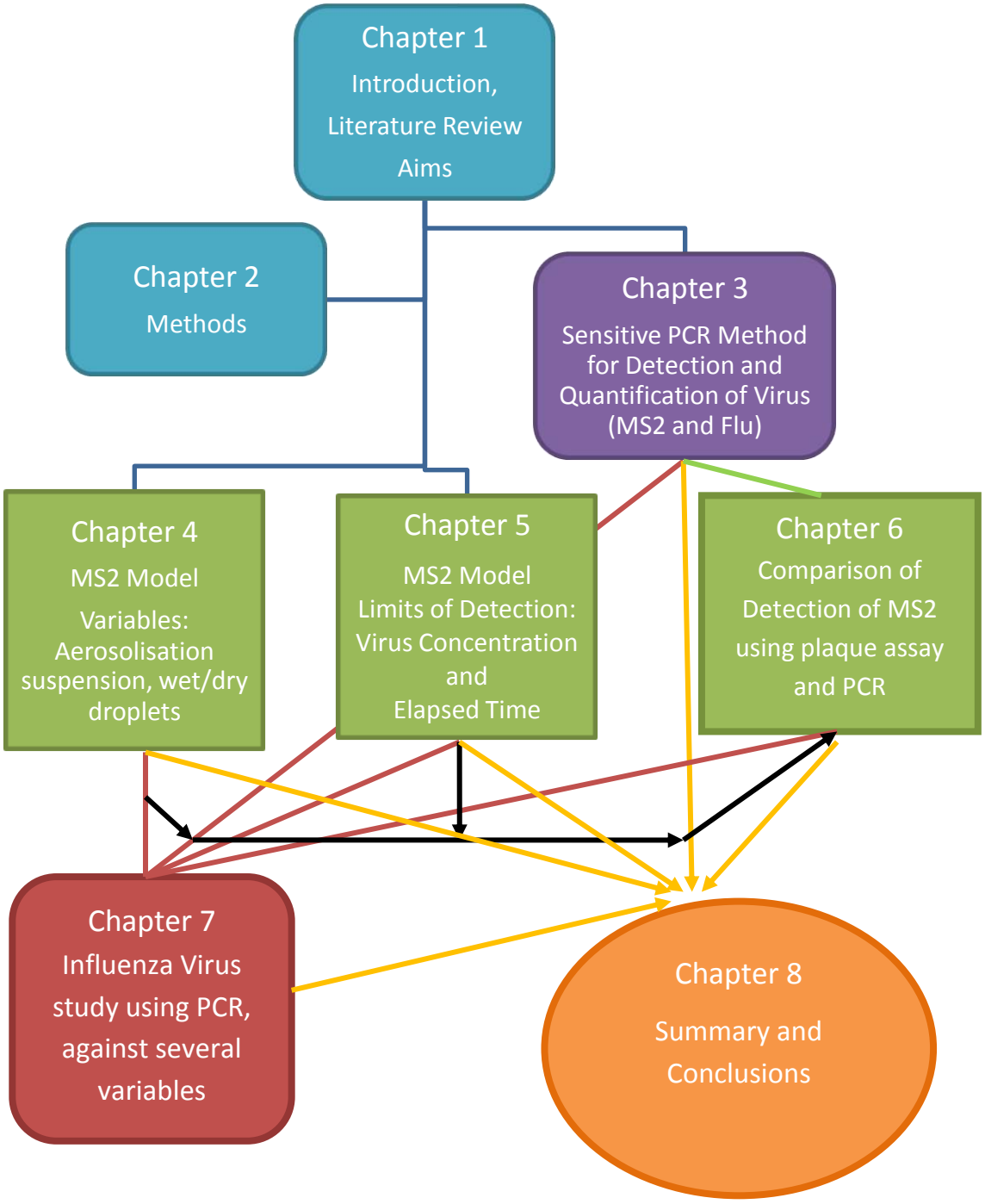
The final aim was to apply knowledge gained from the bacteriophage experiments in the second aim to similar experiments with influenza. The sensitive PCR assay for influenza was used to detect the viruses, as culture techniques are unsuitable for an environmental study of human viruses.

1.4 Account of scientific progress linking the chapters

The first aim of this thesis, development of the sensitive PCR assay for the viruses, is addressed by Chapter three, which is a published article. This describes the design of the assays, validation of the assays, and finally preliminary application of the MS2 assay to some air samples from experiments where MS2 was aerosolised.

The second aim of the thesis is addressed by chapters four, five and six. This work is an exploration of many variables which influence the fate of MS2 in aerosols in simulated experiments. Chapter four describes MS2 recovery in four different types of droplets, by quantification of "culturable" viruses. The fifth Chapter examines recovery of MS2 with variables of elapsed collection time and concentration of viruses in the nebulising suspension, determining the amount of culturable virus. Chapter six compares the detection of MS2 in droplets by the plaque assay and the nested real-time PCR assay, to offer a comparison of the two methods. All chapters examine the differences between liquid nebulising suspensions and the mucous-like nebulising suspension in virus integrity and recovery.

The third aim, examining aerosolisation of influenza from a mucous-like nebulising suspension, is addressed in the seventh Chapter, and describes the application of some limiting factors to aerosolised influenza, based on results from the MS2 experiments. These objectives can be visualised in the following diagram.


Chapter 1
Introduction,
Literature Review
Aims
Chapter 2
Methods
Chapter 3
Sensitive PCR Method for Detection and Quantification of Virus (MS2 and Flu)
Chapter 4
MS2 Model
Variables:
Aerosolisation suspension, wet/dry droplets
Chapter 5
MS2 Model
Limits of Detection:
Virus Concentration
and
Elapsed Time
Chapter 6
Comparison of Detection of MS2 using plaque assay and PCR
Chapter 7
Influenza Virus study using PCR, against several variables
Chapter 8
Summary and Conclusions

Chapter 2: Methods (General)

2.1 Abstract

This chapter describes the general methods used throughout the study. Methods described here include: bacteriophage culture and assay techniques; aerosol testing techniques, including the set-up of the equipment and the generation, collection and testing of the droplets; sample processing; and quantification of samples using PCR and a set of standards. The parameters explored in this study, and the statistical methods used to explore the data, are also described.

2.2 Bacteriophage techniques

2.2.1 Preparation of agar plates with a host overlay

Tryptone agar with Streptomycin and Ampicillin (TASA) plates were prepared by the media staff at Queensland Forensic and Scientific Services (QFSS). The agar was added to a concentration of 1.2 % weight/volume (w/v) (BD Diagnostics, North Ryde, Australia) and the tryptone was added to a concentration of 2 % (w/v) (Oxoid, Thebarton, Australia). The agar also contained streptomycin and ampicillin (Oxoid, Thebarton, Australia) each at a final concentration of 0.0003 % w/v.

The overlay technique employed a second layer of soft TASA agar containing the host culture, onto which the bacteriophage could be subsequently seeded. The soft agar was prepared just as for the TASA agar but with half the amount of agar (0.6 % w/v). To prepare the host culture, 5 mL TBSA (a tryptone soy broth containing streptomycin and ampicillin, with a final concentration of 0.0006 % w/v, developed by the media department at QFSS) was aseptically transferred into a sterile 50 mL falcon tube (Sarstedt, Nümbrecht, Germany), and inoculated with 0.1 mL of the *E. coli* host culture. This culture was incubated at (37 ± 0.1) °C on an orbital shaker (Edwards Instrument Co., Narellan, Australia), run at 300 rotations per minute (rpm) for 16 hours overnight. The following day, the host was subcultured in another falcon tube containing 5 mL TBSA, and incubated for a further three hours with shaking at 300 rpm to permit the host *E.coli* to reach log phase. Molten agar overlays were prepared by melting in a microwave, and then were allowed to cool to 48 (±

0.1) °C in a water bath (Grant, Cambridgeshire, UK). The prepared, log phase *E. coli* culture (approximately 10^9 CFU) was pipetted into an appropriate amount of the soft, molten agar (about 1 mL culture to 450 mL molten agar). The precise temperature of the molten agar was very important; if the agar was even a few degrees cooler it would start to set, and if it was too hot, it killed the cultures. The overlay was poured onto the TASA plates (approximately 12 mL per plate) and then placed in an incubator (Thermoline Scientific, Smithfield, Australia) set to (37 ± 1) °C for one hour to allow the *E. coli* host to recover and form some confluence before being used in experiments.

2.2.2 Bacteriophage propagation

To propagate MS2 bacteriophage, TBSA was used. An MS2 culture and its host, *E. coli* F-amp, were obtained from the Bacteriology department at QFSS. These were originally acquired from ATCC, and the respective ATCC culture numbers were 15597-B1 and 15597. The phage was propagated according to the method of Adams (1959), with some modifications, as detailed below.

A large amount of MS2 phage was initially propagated to store for a culture collection, to be used during the aerosol experiments. This large stock amount was aliquoted out into 1.5 mL volumes, which was deemed sufficient for individual use in experiments, and frozen in 10 % glycerol at -80 °C. To grow a large amount of the phage, 50 mL of TBSA was aseptically transferred into a sterile 250 mL Schott bottle, and inoculated with 1 mL of the *E. coli* host culture. This inoculum was incubated at (37 ± 0.1) °C on an orbital shaker, run at 300 rpm for 16 hours overnight. The following day, the host was subcultured in another bottle containing TBSA, and incubated for a further three hours with shaking at 300 rpm to reach log phase. After this three hour incubation, 1 mL of the revived phage was added to the host culture and incubated for a further two hours at (37 ± 0.1) °C with shaking at 150 rpm. Following this period, the culture was transferred into falcon tubes and centrifuged at 1200 rpm (Beckman Coulter, Brea, US) for ten minutes to pellet the bacteria. The supernatant containing the MS2 was filtered with a 0.2 μm syringe filter with a Supor® membrane (Pall, Port Washington, US) to remove remaining debris.

To determine the bacteriophage titre following propagation, a ten-fold dilution series of the phage was prepared, and dilutions 10^{-7}, 10^{-8} and 10^{-9} were assayed by the overlay technique described in Section 2.1.1 (Adams, 1959). The main difference was that the molten agar was aliquoted into 10 mL tubes before the host culture was added (0.1 mL host culture to each tube). 0.5 mL of each of the dilutions to be assayed was added to each

of three tubes of molten overlay agar, gently swirled, and then immediately poured onto a TASA plate (nine plates in total). The plates were incubated overnight at (37 ± 1) °C, and plaques were counted the following day. An average count of plaques was calculated.

2.3 Aerosol techniques

2.3.1 Chamber set up

A Perspex chamber of approximately 150 L was used for all air testing experiments. It measured 95 cm x 41 cm x 40 cm, and one side was removable for access to the chamber. The inlet and outlet were at opposite ends of the chamber. The set up is displayed in the schematic diagram (see figure 2-1).

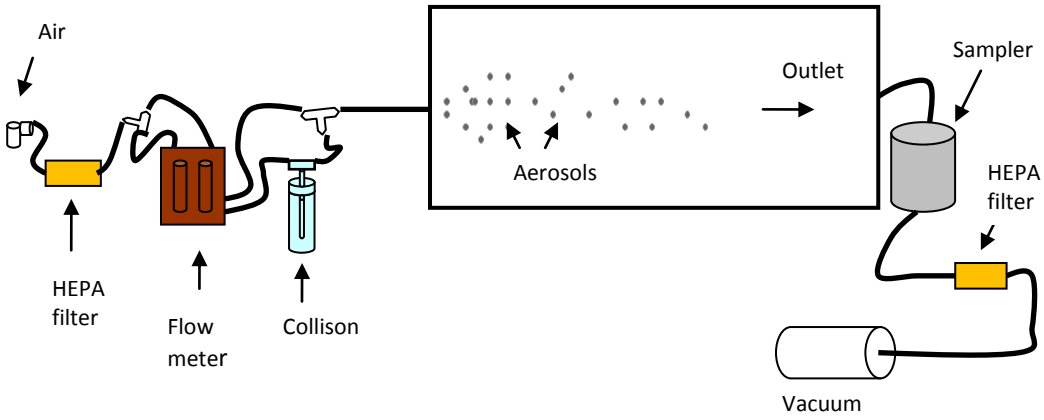


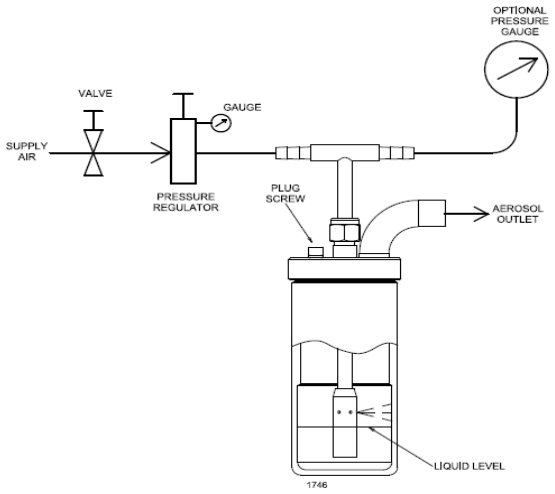
Figure 2-1 Diagram of chamber set up used for all aerosol experiments

2.3.2 Aerosol delivery

Aerosols were created and delivered into the chamber with the Collison six-jet nebuliser (BGI Inc, Waltham, US). This is the most commonly used nebuliser in studies generating and measuring droplets (see fig 2-2). A Precious Fluids Jar was used in conjunction with the Collison, which allows the use of a small volume of nebulising suspension (down to 5mL), so that the concentration of the viruses would not be too diluted in the suspension. The size of the aerosol particles is determined by the pressure of the air passing through the Collison. This was regulated using an air flow metre (Cole Parmer, Vernon Hills, US) which maintained a flow rate of six litres per minute (L/min), the air flow required to produce

aerosols with a size of around 2 µm. Aerosols of other sizes may still be produced, but this is not a problem as these experiments were designed to simulate respiratory droplets, and naturally occurring droplets are never of a uniform size. An air tap was used, and first passed through a HEPA filter (Pall, Port Washington, US) to ensure that air entering the Collison was sterile.

The droplets were delivered into the chamber in two different forms: the first type were delivered directly into the chamber from the Collison, and are referred to as 'wet droplets'. The second type of droplet was mixed with sterile air at about 12 L/min, in order to dry the droplets before they were delivered into the chamber. These were referred to as 'dry droplets'.



2-2 Schematic diagram of the six-jet Collison nebuliser (taken from BGI manual)

2.3.3 Aerosol collection

Air was sampled from the chamber using impactor samplers. Two types were employed for the two methods of analysis: a six stage Andersen impactor (Thermo Electron Corporation Waltham, US) for the plaque assays and a BioStage® single stage viable cascade impactor (SKC Inc., Eighty Four, US), for the samples on which PCR analysis was to be undertaken. The six stage sampler was used for the plaque assays because it was desirable to determine if the recovery of intact viruses was affected by droplet size. TASA overlay plates were used in this sampler. For the experiments where samples were to be analysed by PCR, it was not appropriate to use the six-stage sampler as that would have produced 18 samples per experiment, as opposed to three. In addition, the six stage sampler would have been very

cumbersome for working with liquids such as buffers added to the agar plate impaction surfaces, as described in the following paragraph.

The BioStage® single stage sampler was used in experiments to be analysed with PCR so as to decrease the number of samples to analyse, saving on both time and cost. It collects droplets ≥0.6 µm. To our knowledge, this particular sampler has not been used to collect viruses for PCR analysis before, so it was necessary to develop a suitable collection protocol. The protocol we developed for this study used agar plates containing non-nutritional agar in the sampler, overlayed with a layer of phosphate buffered saline (PBS) with a pH of 7.4 (provided by the media department, QFSS) to collect the viruses. A small amount of PBS (1.8mL) was used to collect the sample, to minimise the chance of splashing of the buffer inside the sampler during impaction, and also to ensure that the sample was not too dilute to process. The buffer was collected following each replicate, to be used for RNA extraction and PCR analysis. Trial experiments showed our method to be successful in collecting the virus and allowing both RNA extraction and PCR analysis.

Both samplers were connected to a specialised vacuum pump (Thermo Electron Corporation, Waltham, US) which had a flow rate of 28.3 L/min. This meant that a sampling time of 5.5 mins was required to sample all of the air in the chamber (155.65L). The output vent of the samplers was connected to a HEPA filter (Pall, Port Washington, US) to ensure that no contaminated air was allowed into the vacuum pump or laboratory air.

2.3.4 Preparation of materials for aerosol experiments

Before each experiment, a tube of stock MS2 or influenza was taken out of the -80 °C freezer and allowed to thaw. The nebulising suspensions were prepared by diluting the viral stocks in the nebulising suspension (PBS or artificial mucous). PBS was obtained from the media department and artificial mucous preparation (0.4 %) was based on the method of King (King *et al.*, 1985). 0.4 g of locust bean gum (LBG; Sigma Aldrich, St Louis, US) was dissolved in 100 mL boiled water, with mixing, and allowed to cool. This concentration was chosen because higher concentrations of the LBG produced a heavily viscous suspension that blocked the Collison nebuliser.

The nebulising suspension and dilution of the virus in the suspension depended on the variable being tested in the given experiment. The nebulising suspension was kept on ice during the experiment so as to minimise the degradation of the viruses in the suspension. All experiments were conducted in triplicate, and in between each replicate, the nebulising

suspension was replaced in the jar, so that the viruses were fresh for each replication, and not constantly undergoing aerosolisation.

2.3.5 Experimental parameters and variables

Aerosolisation and sampling parameters were altered according to differing variables, as follows.

A 'standard' experiment was the baseline experiment, which was used to compare all changing variables. The sampling variables, which were explored in experiments, were the concentration of the nebulising suspension, and the 'elapsed time' delay (between beginning of delivery and beginning of sampling). As constants, these variables were held at:

- Concentration of the nebulising suspension – 10^5 PFU/mL
- Elapsed time – 0 minutes

Other variables explored in the aerosolisation experiments were: the type of droplet – wet or dry; the detection method – plaque or PCR; and the type of nebulising suspension – PBS or artificial mucous. Standard experiments were performed for each of these variables as well, with only one variable changing at a time. All experiments were performed in triplicate, i.e. for each changing variable, three aerosolisations were performed, and one sample taken for each replicate, giving three samples per experiment.

2.3.6 Sample processing

2.3.6.1 Plaque assay processing

Following the experiments which used TASA overlay plates to collect the aerosolised phage, the TASA plates were taken out of the sampler and immediately put into an (37 °C ± 0.1 °C) incubator (Contherm Scientific Ltd., Wellington, NZ), for overnight incubation. Plaques were counted the following day, and typically appeared as small 'holes' in the host lawn, about 1-2 mm in diameter. The relative recovery of the plaques were expressed as a percentage of virus originally challenged in the chamber; this was estimated by measuring the amount of nebulising suspension before and after each replicate, by weight, to determine how much suspension was aerosolised into the chamber, and thus how many viruses were present in the challenge. This is shown by the following equation:

$$relative\ recovery\ (\%) = \frac{P_r \times (1/V_a)}{P_i} \times 100$$

Where P_r= plaques recovered and corrected using positive-hole correction table, V_a = volume aerosolised (mL), P_i = no. of plaques in 1 mL of the initial nebulising solution

2.3.6.2 Nested real-time PCR assay processing

We initially trialled the collection of viruses into a layer of RNA lysis buffer in a petri dish, which was first washed with fetal calf serum to prevent binding of the viruses to the plastic. However, we decided against this method because the use of lysis buffer in this application was not well documented in comparison with other types of buffer such as PBS or peptone water. PBS Buffer (pH 7.4) was selected for the remaining steps because its use was well documented and readily available. Additionally, the Andersen cascade sampler requires a particular height of agar in the petri dishes for efficient collection of droplets, and there was no documentation available on the combined use of lysis buffer and agar.

Buffer collected from agar plates for RNA extraction was measured by weight to determine the sample size; this was necessary because for the majority of experiments, a 140 µL sample (rather than the entire sample) was extracted and analysed. For experiments where the amount of virus collected was expected to be quite low, RNA was extracted from most of the sample or from the entire sample. All of the RNA extracts were stored in the -80 °C freezer and analysed by PCR together at the end of experimentation. For experiments where the expected yield was unknown or minimal, the samples were screened first in order to determine if RNA was present. If no RNA was observed, the experiments were repeated, with whole samples analysed to maximise detection of the virus.

RNA extraction was performed with QIAGEN's RNeasy extraction kit (QIAGEN, Doncaster, Australia), using the centrifuge method, as per the manufacturer's instructions. Briefly, 140 µL of collected buffer containing RNA (or entire sample depending on predicted outcomes of the specific experiment) was pipetted into 560 µL AVL lysis buffer, vortexed for 15 seconds and left at room temperature for ten minutes. 560 µL of 100 % ethanol was then added to the tube and vortexed for 15 seconds. The RNA was pipetted onto a spin column and centrifuged at 8,000 rpm for one minute. The collection tube was replaced, 500 µL of wash buffer AW1 was pipetted onto the column, and the tube was centrifuged at 8,000 rpm for one minute. The collection tube was again replaced, and 500 µL of wash buffer AW2 was pipetted onto the column, then centrifuged at 14,000 rpm for three

minutes. The column was placed into a new collection tube and centrifuged for a further one minute. The column was then placed into a sterile 1.5 mL tube, 60 µL of AVE buffer was pipetted onto the column and it was incubated for one minute. The tube was then centrifuged at 8,000 rpm for one minute to recover the RNA into the tube, and stored at -80 °C. Bovine diarrhoea virus (BVDV) was used as a positive extraction control, and water was used as a negative extraction control.

2.4 Quantification of samples by nested real-time PCR

The design of the nested real-time PCR assays is described in detail in Chapter three. Below is a description of the construction of the standard curve which was used to estimate the concentration of viruses in the collected air samples. Synthetic RNA was used to construct the standard curves, as it was considered a more suitable option than extracted RNA. RNA extracted from culture preparations contains extraneous nucleic material which occurs naturally. This material may lead to inaccurate quantification, as we are only interested in the RNA inside intact viruses, and not the extra-virion RNA. Synthetic RNA was transcribed from a DNA template as described in the following sections, based on the method described by Smith and colleagues (2006), whereby synthetic DNA and RNA were prepared for use as controls in diagnostic assays.

2.4.1 Preparation and ligation of oligonucleotides

The designed synthetic standards were essentially the target of the nested real-time PCR assays; for MS2 this was a 188 bp fragment, and for influenza it was a 125 bp fragment. These sequences had to be constructed from shorter oligonucleotides, as it is generally not possible to purchase oligonucleotides greater than 100 bp in length. In addition to the viral target sequences, the controls needed to have the T7 transcription sequence on the 5’ end, followed by a few bases to ensure none of the target sequence was lost in any of the genetic manipulation processes (sequences can be found in appendix B). The oligonucleotides were designed so that they would ligate to form DNA linkers with sticky ends (with six overhanging bases), as illustrated in figure 2-3.

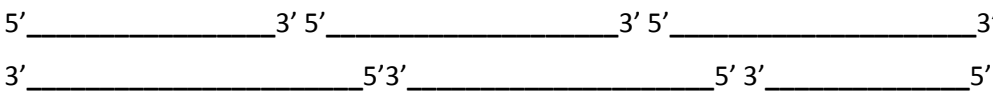


Figure 2-3 Diagram representing the design of the MS2 oligonucleotides obtained from Geneworks, which were ligated to form one DNA strand. The oligonucleotides were named F1, F2 and F3 (5’-3’) on the forward strand and R1, R2 and R3 (5’-3’) on the reverse strand. The influenza oligonucleotides were designed similarly, however only two oligonucleotides for each strand were required as the entire sequence (150 bp) was shorter than that of the MS2 sequence (214 bp). They were named F1 and F2, and R1 and R2.

The MS2 synthetic control was 214 bp in total length (including the T7 sequence), and sectioned into six oligonucleotides of around 67-76 bp (three each for the forward and reverse strands); the influenza control was 150 bp in length (including the T7 sequence), and four oligonucleotides (two each for the forward and reverse strands) of around 68-82 bp were designed. Oligonucleotides were obtained with HPLC purification (Geneworks, Hindmarsh, Australia).

T4 ligase will only ligate DNA which has phosphorylated 5’ ends, which the oligonucleotides did not possess, as the HPLC purification step does not accommodate this modification. A T4 polynucleotide kinase (PNK) kit (Promega, Alexandria, Australia) was used to phosphorylate the ten oligonucleotides. ATP was purchased separately from Astral Scientific (Caringbah, Australia). Phosphorylation was performed, as per the manufacturer’s instructions, by adding the kit components of 10X buffer, ten units of T4 PNK, DNase-RNase-free water, ATP and the oligonucleotide in a 40 µL reaction, and incubating it for ten minutes at 37 °C. The reactions were stopped by adding 0.5 M EDTA, and cleaned up by standard phenol extraction.

Following phosphorylation, the oligonucleotides had to be annealed into DNA linkers with sticky ends. The oligonucleotides which were designed as aligned pairs (as seen in figure 2-3, e.g. F1 and R3 were aligned) were put into tubes in equimolar concentrations (40 pmol/µL), and heated to 94 °C for two minutes to separate the strands. The tubes were left to cool to room temperature for ten minutes to allow annealing of the pairs into linkers. The oligonucleotides, now prepared for ligation, were ligated using a T4 ligase kit as per the manufacturer’s instructions (Roche, Manheim, Germany). The reactions contained 10X ligation buffer, DNase- RNase-free water, T4 ligase (1 U) and the template DNA linkers (approximately 500 ng in one reaction). The reactions were incubated overnight at 4 °C, and stopped by heating to 65 °C for ten minutes, to deactivate the enzyme.

It was then necessary to produce double-stranded DNA (dsDNA) and to amplify the products, as they were in low concentrations. High Fidelity *Taq* mastermix was obtained from Invitrogen (Mulgrave, Australia), because it was important to retain the integrity of the DNA sequence. 2 µL of the ligation reaction was pipetted into 48 µL mastermix to give a 50 µL reaction, and the tubes were amplified on an Eppendorf ep Gradient S Mastercycler (Eppendorf, Hamburg, Germany) using an initial denaturation step of 94 °C for two minutes, followed by 40 cycles of 94 °C for 15 seconds, 59 °C for 15 seconds and 68 °C for 30 seconds. This was followed by a further elongation step of 68 °C for two minutes. Primers designed specifically for this purpose were obtained (see appendix B). They were based on the sequences of the two whole oligonucleotides (MS2 and influenza), with appropriate melting temperatures as per normal parameters. The PCR products were electrophoretically separated using Bio-Rad apparatus (Bio-Rad Laboratories, Gladesville, Australia) to ensure that the products were the right size. Electrophoresis was undertaken at 100 volts for 45 minutes, using a 100 bp marker (Promega, Alexandria, Australia) to check the size of the PCR products. PCR products were stained with ethidium bromide (Astral Scientific, Caringbah, Australia) and visualised under UV light using a Gel Doc XR+ system (Bio-Rad Laboratories, Gladesville, Australia).

DNA was quantified by UV spectrophotometry, using the Nanodrop 2000C (Thermo Fisher, Scoresby, Australia). The output gave a full spectrum, and also noted the 260:280 and 260:230 absorbance ratios, all of which were close to the ideal values for DNA (1.8 and 2.0 respectively). Water was used as a reference.

2.4.2 Transcription from DNA template

Transcription was performed with the Riboprobe® in vitro Transcription System (Promega, Alexandria, Australia), using the prepared dsDNA templates. The reactions were prepared as per the manufacturer's instructions: each reaction had a volume of 20 µL, and contained transcription buffer, T7 transcriptase, DTT, ribonucleotides rATP, rGTP, rCTP and rTTP, the DNA templates, and DNase- RNase-free water. The transcription reactions were incubated at (37 ± 0.1 °C) for one hour. DNase treatment was performed to remove the DNA templates; this involved two successive treatments with one unit of DNase 1 and a 15 minute incubation, to ensure maximum removal. Then a chloroform and ethanol extraction was performed, using 1.5 mL heavy phase lock gel tubes (5 PRIME, Hamburg, Germany) to separate the phases, so the aqueous layer containing the RNA was preserved. After the DNA removal step, the remaining RNA was filtered through a 30 K Amicon® Ultra 0.5 mL

centrifugal filter (Millipore, NSW, Australia) to remove any unincorporated nucleotides; it was important to ensure there was minimal nucleic acids, other than the desired target RNA.

Spectrophotometric analysis of the RNA was used to estimate quantity and quality of the nucleic acid. A full spectrum was obtained from the Nanodrop 2000C, as well as the ratios mentioned above in Section 2.4.1. The calculated ratios were 2.0 or above (the ideal ratio for RNA). Water was again used as a reference.

Once the quantities of RNA were known, the stock was diluted into a ten-fold series, aliquoted into screw-cap tubes and stored at -80^{o}C.

2.5 Data Analysis

SPSS (v. 17.0) statistical software (SPSS Inc., Chicago, US) was used to analyse all of the data. A Chi-squared test was used to determine whether the virus-laden droplets had the same size distribution as the mean droplet sizes generated by the Collison, by goodness-of-fit. Two-way analysis of variance (ANOVA) was used to determine whether there was a change in the infectivity of viruses in different suspensions (conducted in Chapter four). Two-way ANOVA tests were used to compare the mean bacteriophage recoveries from: wet and dry droplets; each type of suspending medium; and the variables investigated. For the influenza study, two-way ANOVA tests were used to compare the means of the viruses detected from the two types of droplets examined.

Chapter 3: A nested real-time PCR assay has an increased sensitivity suitable for detection of viruses in aerosol studies

3.1 Abstract

Influenza is commonly spread by infectious aerosols, generated from infected persons. However, detection of viruses in aerosols is not sensitive enough to confirm the characteristics of virus aerosols. The aim of this study was to develop an assay for respiratory viruses sufficiently sensitive to be used in epidemiological studies.

To achieve this aim, a two-step, nested real-time PCR assay was developed. Assays were developed for MS2 bacteriophage, used as a surrogate organism for influenza in aerosol studies, and for the respiratory viruses influenza A and B, parainfluenza 1 and human respiratory syncytial virus. Outer primer pairs were designed to nest each existing real-time PCR assay. The sensitivities of the nested real-time PCR assays were compared to those of existing real-time PCR assays. Both nested real-time PCR assays were applied in an aerosol study to compare their abilities to detect bacteriophage in air samples.

The nested real-time PCR assays were found to be several logs more sensitive than the real-time PCR assays, with lower levels of virus detected at lower Ct values. The nested real-time PCR assay successfully detected MS2 in air samples, whereas the real-time assay was unable to do so.

The sensitive assays for respiratory viruses will permit further research using air samples from artificially and naturally generated virus aerosols. This data will inform current knowledge regarding the fate of respiratory viruses and risks associated with the spread of viruses through aerosol transmission.

3.2 Introduction

Influenza is a common respiratory illness occurring in seasonal epidemics. While it is normally a mild infection, it has the potential to be severe, particularly in the young, elderly and immunocompromised. There are three routes of infection: transmission by direct and

indirect contact, and aerosol transmission, which is defined by the expulsion of fine, infectious particles into the atmosphere by respiratory activities such as coughing, sneezing or talking (McCluskey *et al.*, 1996). Whilst direct transmission and indirect transmission are relatively well documented, little is known about aerosol transmission of viruses like influenza.

The lack of knowledge on this topic can be mainly attributed to the deficiency of suitable detection systems, and poor integration of biotechnology with aerosol science (Peccia and Hernandez, 2006). Traditionally, studies investigating infectious aerosols have been culture-based. Whilst this can provide useful information on infectious potentials of micro-organisms, there are fundamental problems and limitations with culture techniques which can consequentially underestimate the quantity of bioaerosols in the atmosphere. This is particularly the case with airborne viruses.

Infectivity, or viability, is a significantly limiting factor when using culture techniques. As infectivity is negatively affected by collection forces associated with air samplers, and the use of nebulisers in simulated studies, culture techniques will always give underestimates of the actual count of micro-organisms in the atmosphere. In addition to this, it is possible that not all infective viruses can initiate a plaque, thus compounding false negative results.

It is difficult to quantify the proportion of microorganisms which remain infectious, which would indeed vary for different organisms, but some studies have reported that fewer than 1% of bacteria from terrestrial and aquatic environments could be recovered (Amann *et al.*, 1995, Pace, 1997). Similar, and more exacerbated findings could be anticipated for studies with airborne viruses, which have much more complex culture requirements than bacteria. Traditional cell culture for human viruses is simply not compatible with current air sampling techniques, and thorough studies on the airborne spread of human viruses, using cell culture, has not been possible due to the inability of many viruses to be cultured (Sattar and Ijaz, 1997). Limitations of culture techniques include long processing times; limited dynamic range of one log per plate; susceptibility of media to environmental conditions, resulting in decreased infectivity; potential error in visual counts; and procedural errors (Edelman and Barletta, 2003). Furthermore, probable contamination issues brought about by the nature of aerosol experiments, for both simulated and field studies, compound the problems of cell culture for isolation and detection of viruses in aerosols. Such experimental conditions, to which the cultures would be subjected, may include being left for delayed periods of time, and remaining exposed in non-sterile areas. Contamination and environmental exposure compromises the integrity of the results of aerosol studies.

The application of molecular methods, such as PCR, has the potential to be much more suitable for aerosol studies, and indeed has become more common in recent years. Some advantages of PCR in aerosol science include relative simplicity of sample processing; increased accuracy, processing time and sample throughput; greater dynamic range (over 7 logs); and perhaps most importantly, increased sensitivity (Peccia and Hernandez, 2006). Theoretically, PCR can detect as little as one organism, and such detection levels have been demonstrated using real-time PCR (Rose *et al.*, 1997, Edelman and Barletta, 2003). Another distinct difference between culture and PCR is that PCR is independent of infectivity. Although this means that PCR can give no information regarding the amount of infective viruses in a sample, it does overcome the problem of loss of infectivity as a result of air collection (Peccia and Hernandez, 2006). Some studies have performed statistical comparisons of the results of plaque assays and PCR, and one found there was a significant correlation (Edelman and Barletta, 2003). As yet, there is no established 'gold standard' PCR technique for detection of viruses from aerosols, and it would seem that each method should be tailored to the intended purpose and virus in question. Some published studies have used conventional PCR (Agranovski *et al.*, 2006), and semi-nested or nested PCR (Myatt *et al.*, 2003). Others have used real-time PCR (Schweigkofler *et al.*, 2004, Zeng *et al.*, 2004).

Thus the aim of this study was to develop a suitable assay for the purposes of examining the distribution of human respiratory viruses in air samples, and also an assay for a surrogate of such viruses, MS2 bacteriophage. Due to the high probability of viruses existing in naturally produced aerosols in low numbers, it is necessary to design a highly sensitive assay. Respiratory virus load in human infections is likely to vary widely, as the amount of virus shed is high in the initial days of infection, but lower in later days of infection.

The purpose of this study was to develop a particularly sensitive assay by combining nested PCR with existing real-time PCR assays, all using TaqMan® chemistry, to create a nested real-time PCR. Nested real-time PCR is not common, but has been previously described for animal clinical samples or viruses cultured from clinical samples (Petrik *et al.*, 1997, McGoldrick *et al.*, 1998, Heath *et al.*, 2003, Wu *et al.*, 2004), and would theoretically allow both an increased sensitivity as well as quantification of viruses in the air samples. There has been no previous application of similar methods to environmental sampling.

3.3 Materials and Methods

3.3.1 Selection of organisms

In this study, nested real-time PCR assays were developed for common respiratory viruses including influenza A, subtypes H1N1 and H3N2, influenza B, parainfluenza 1 and human respiratory syncytial virus. A similar nested real-time PCR assay was also developed for MS2, a bacteriophage which has been used as a model for airborne viruses including influenza (Foarde *et al.*, 1999, Barker and Jones, 2005, Hogan *et al.*, 2005). Bacteriophages are commonly used as surrogates because they are non-pathogenic to humans and are easy to propagate in the laboratory (Barker and Jones, 2005).

3.3.2 Propagation of virus and RNA extraction

Viral stocks of the respiratory viruses, from Queensland Health Forensic and Scientific Services, were used to test the assays. $TCID_{50}$ assays were performed to determine the titre of each virus.

Bacteriophage was propagated in broth culture with its *E. coli* F-amp host according to the method of Adams (1959) with the some modifications (Adams, 1959). Briefly, the host was cultured overnight in a tryptone broth containing streptomycin and ampicillin, after which it was subcultured and incubated for a further 3 hours. 1mL rehydrated phage was added to the culture which was incubated for a further 2 hours, and then centrifuged at 1200 rpm for 10 mins to pellet the bacteria. The supernatant containing the MS2 was filtered with a 0.2 µm filter to remove remaining debris.

To determine the bacteriophage titre, a 10-fold dilution series of the phage was prepared, and dilutions 10^{-7}, 10^{-8} and 10^{-9} were assayed by the overlay technique (Adams, 1959). 0.5 mL of each dilution was added to 3.5 mL of molten 0.6 % tryptone agar (soft agar) containing *E. coli*, which was poured over the surface of tryptone agar (TASA) plates. The plates were incubated, and the number of plaques per plate were counted and averaged to give a titre in plaque forming units per mL (PFU/mL).

Each viral stock was prepared in a 10-fold dilution (for 7 logs) series for testing of sensitivity of the assays. Viral RNA of each dilution was extracted using the Qiagen RNeasy Viral mini kit, as per the manufacturer's instructions.

Throughout the study, the virus and bacteriophage concentrations have been converted to reflect the number of virions or RNA copies present in each reaction, which is expressed as

$TCID_{50}$ (50% tissue culture infectious dose) or PFU (plaque forming units), for viruses and bacteriophage respectively.

3.3.3 Design of primer sets for nested real-time PCR

Primer sets for the nested real-time PCR assays were designed based on previously described TaqMan® assays for each virus (see table 3-1). Using Primer Express®, an outer primer pair was designed to nest each TaqMan® primer/probe set as shown in figure 3-1. This outer primer pair was used in the first round of amplification, which included a reverse transcription step (RT-PCR), followed by a second round of amplification using the TaqMan® primers and probe.

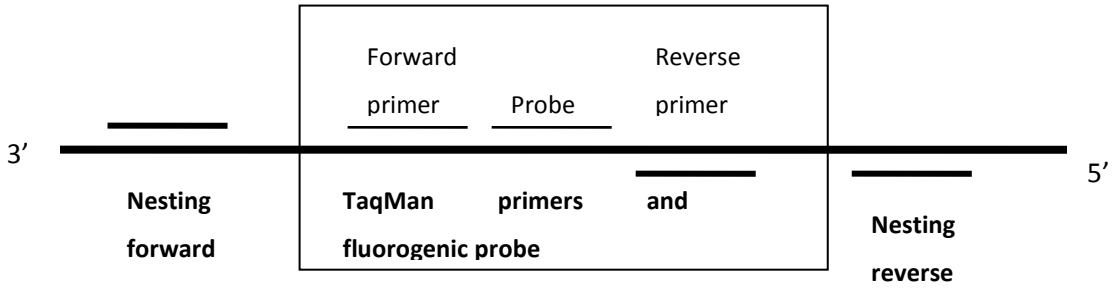


Figure 3-1. This diagram displays the positioning of the primer sets designed in this study, shown in relation to the existing TaqMan® primers and probe. The new primer sets were designed to nest the TaqMan® target sequences. This was done for each of the viruses in this study.

3.3.4 Real-time PCR conditions

For the previously described real-time PCR assays, Universal mix (Applied Biosystems) was prepared by adding primers and probe to the reaction mix at varied final concentrations (final concentrations differed for each assay; see table 3.1) in a 20 µL reaction volume. 5 µL of PCR product was added to each reaction mix. The reactions were performed on an ABI 7500 system, with the cycling conditions of 50 °C for 30 mins (RT step) followed by 95 °C for 10 mins, and then 40 cycles of 95 °C for 15 secs and 60 °C for 60 secs.

3.3.5 Nested real-time PCR conditions

The assay was performed in two steps; first round RT-PCR and second round real-time PCR. For the first round of amplification, SuperScript™ III One-Step RT-PCR with Platinum® Taq DNA polymerase mastermix (Invitrogen, Vic., Australia) was prepared by adding SuperScript™ III RT /Platinum® Taq Mix to the 2x reaction mix (50 µL final volume).

Table 3-1. All primers and probes for each virus used in this study are shown in this table. This includes the outer (or nesting) primer pairs as well as the existing TaqMan® primers and probes.

Organism	Reference	Primer set	Primer name	Sequence (5' -3')	Final concentration (pmol/µL)
Influenza A (H1N1)	This study	Outer	FluAH1-F	TCGCACAGAGACTTGAAGATG	0.4
			FluAH1-R	CGTGAACACAAATCCTAAAATC	0.4
		Inner	FluF	AGGCTCTCATGGARTGGCTAAA	0.6
			FluR	AAACCCTAAAATCCCCTTAGTCAGA	0.6
		Probe	FluP	CAAGACCAATCCTGTCAC	0.048
Influenza A (H3N2)	This study	Outer	FluA-F	TCGCGCAGAGACTTGAAGATG	0.4
			FluA-R	CGTGAACACAAACCCCAAAATC	0.4
		Inner	FluF	AGGCTCTCATGGARTGGCTAAA	0.6
			FluR	AAACCCTAAAATCCCCTTAGTCAGA	0.6
		Probe	FluP	CAAGACCAATCCTGTCAC	0.048
Influenza B	This study	Outer	FluB-F	TGCCTCCACGAAAAATACGG	0.4
			FluB-R	CCTGCAATCATTCCTTCCCA	0.4
		Inner	INFB-1	AAATACGGTGGATTAAATAAAAGCAA	0.15
			INFB-2	CCA GCA ATA GCT CCG AAG AAA	0.15
		Probe	INFB probe	CACCCATATTGGGCAATTTCCTATGGC	0.1
Para-influenza 1	This study	Outer	PIV1-F	AGGATGTGCAGATATAGGGAA	0.4
			PIV1-R	GTCTCATTCACAGTGGGCAA	0.4
		Inner	Para1-F	TTTAAACCCGGTAATTTCTCATACCT	0.3
			Para1-R	CCCCTTGTTCCTGCAGCTATT	0.3
		Probe	Para1 probe	TGACATCAACGACAACAGGAAATCATGTTC TG	0.15
Human Respiratory Syncytial Virus	This study	Outer	RSV-F1	TATTTGCATCGCCTTACAGTC	0.4
			RSV-R1	CTAAGGCCAAAGCTTATACAG	0.4
		Inner	RSVF	AGTAGACCATGTGAATTCCCTGC	0.3
			RSVR	GTCGATATCTTCATCACCATACTTTTCTGTTA	0.3
		Probe	RSV probe	TCAATACCAGCTTATAGAAC	0.15
MS2	This study	Outer	MS2-F5O	TGA ACA AGC AAC CGT TAC CCC	0.4
			MS2-R5O	TAT CAG GCT CCT TAC AGG CAG C	0.4
	O'Connell et al., 2006	Inner	MS2F5	GCT CTG AGA GCG GCT CTA TTG	0.4
			MS2R5	CGT TAT AGC GGA CCG CGT	0.4
		Probe	MS2-5 probe	CCGAGACCAATGTGCGCCGTG	0.2

Primers were added to a final concentration of 0.4 pmol/µL. 5 µL of RNA sample was added to the mixture per 50 µL reaction. For the negative control, RNA sample was substituted with nuclease-free water. Assays were performed on a Geneamp® 9700 thermocycler (Applied Biosystems, Vic., Australia) with the cycling conditions of 50 °C for 30 mins (RT step); 20 cycles of 94 °C for 45 secs, 55 °C for 45 secs, 72 °C for 30 secs; then 72 °C for 7 mins. Samples were electrophoretically separated on a 2 % agarose gel, which was visualised with UV illumination to detect the presence of PCR product.

The second round amplification was performed as for the real-time PCR assay, described above. As the two assays were run simultaneously to compare their sensitivities, an RT step was included, although it is not necessary as cDNA is synthesised in first round amplification. The assay was performed on an ABI 7500 system, with the cycling conditions of 50 °C for 30 mins (RT step) followed by 95 °C for 10 mins, and then 40 cycles of 95 °C for 15 secs and 60 °C for 60 secs.

3.3.6 Trial application to air samples

To test the application of the nested real-time PCR to aerosol studies, the assay was carried out on air samples containing aerosolised MS2 phage. A nebulising suspension of MS2 (10^6 PFU/mL) was prepared in PBS and each dilution was subsequently aerosolised with a Collison 6-jet nebuliser, operated at 10 L/min, inside a chamber. Aerosols were collected by gravitational settling onto sterile, empty petri dishes which were previously washed with 10 % fetal calf serum (FCS) in PBS, to prevent binding of the viruses to the plastic. Four variations of the test were performed, each introducing different amounts of the virus into the chamber by varying the aerosolisation time. Four samples were taken from each test. After each sample was collected, the dishes were washed with 560 µL lysis buffer, and collected into tubes. Extraction was then performed as normal using the Qiagen RNeasy Mini kit. The MS2 nested real-time PCR assay was performed on the extracts to determine if the nested real-time PCR assay could detect MS2 in samples where the real-time PCR assay could not. This was done as described above, with the exception that the first round PCR was performed using 40 cycles as opposed to 20 cycles.

During the tests, TASA settle plates were simultaneously used to collect aerosolised virus to determine the recovery of infectious viruses for each experiment. These results were then used as a reference point for the PCR results.

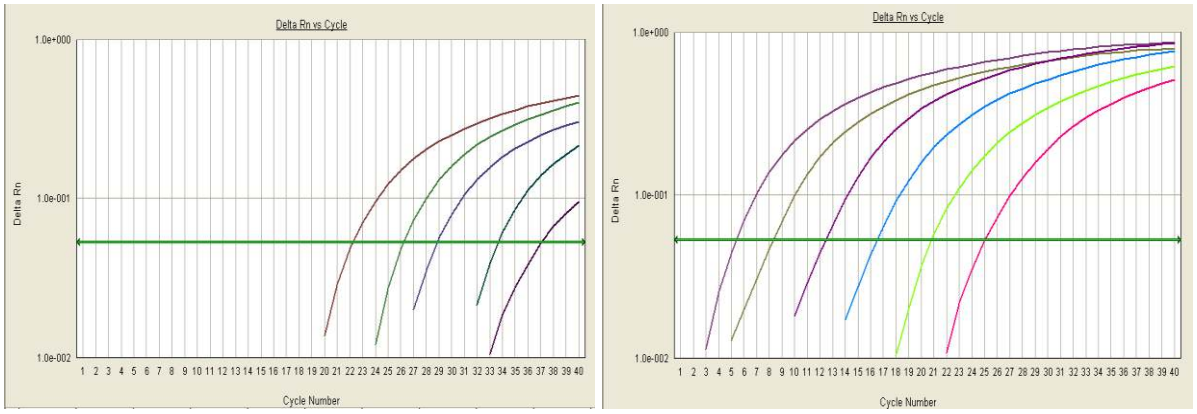


Figure 3-2. This figure shows the comparison of the real-time PCR assay (left) with the nested real-time PCR assay (right) for a 10-fold dilution series of influenza A, subtype H1N1. The concentrations of H1N1 virus tested ranged from 10^4 $TCID_{50}$ down to 0.1 $TCID_{50}$. The nested real-time PCR assay was able to detect 0.1 $TCID_{50}$, a log further than the real-time PCR assay. Additionally, all virus dilutions were detected at much lower Ct values than the real-time PCR assay.

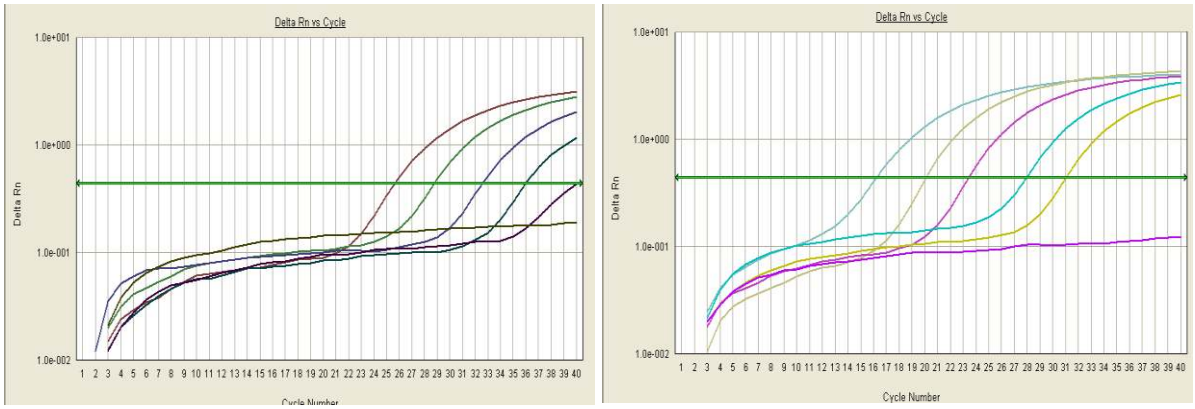


Figure 3-3 This figure shows the comparison of the real-time PCR assay (left) with the nested real-time PCR assay (right) for a 10-fold dilution series of human respiratory syncytial virus (RSV). The highest concentration of RSV tested was from 10^4 $TCID_{50}$ down to 0.1 $TCID_{50}$. The nested real-time PCR assay was able to detect 1 $TCID_{50}$, a log further than the real-time PCR assay. Additionally, all virus dilutions were detected at much lower Ct values than the real-time PCR assay, with all titres detected after 31 cycles of real-time PCR.

3.4 Results

3.4.1 Detection limits and sensitivities of nested real-time PCR assays

The nested real-time PCR assays for all five of the viruses tested in this study were able to detect lower concentrations of viral RNA than the respective non-nested real-time PCR assay. In addition to this, the nested real-time PCR assay amplified products that were detected at earlier Cts than the real-time PCR assays. All respiratory virus results are presented in table 3-2.

The real-time PCR assay for influenza A, subtype H1N1, had an endpoint of 1 $TCID_{50}$, detected at 36.99 Ct (see figure 3-2). The nested real-time PCR assay, detected the same virus concentration at 20.64 Ct. Moreover, it detected a further log dilution of 0.1 $TCID_{50}$, at 25.04 Ct. The 10-fold dilution series using the nested real-time PCR assay was well distributed, with intervals between 2.81 and 4.34 cycles, with samples detected in the range of 5.47 Ct to 25.04 Ct for a virus titre range of 10^4 to 0.1 $TCID_{50}$. In comparison, using the real-time PCR assay, the amplifications were detected only in the last half of the cycle program, starting at 22.17 Ct for 10^4 $TCID_{50}$ and ending at 36.99 Ct for 1 $TCID_{50}$. The virus dilutions were detected by the nested real-time PCR assay at about 17 cycles earlier than by the real-time PCR assay.

Influenza A subtype H3N2 was detected by the real-time PCR assay, starting from 25.80 Ct for the highest dilution of virus, 10^5 $TCID_{50}$. The endpoint of this assay was 34.86 Ct for 100 $TCID_{50}$. In contrast, using the nested real-time PCR assay, the virus was detected at 5.92 Ct for a titre of 10^5 $TCID_{50}$, with an endpoint of 1 $TCID_{50}$ detected at 20.45 Ct. A good distribution of the 10-fold dilutions was observed, with intervals of 2.57-3.39 cycles between each dilution. The nested real-time PCR assay detected the viruses at an average of 19.5 cycles earlier than the real-time PCR assay.

Influenza B was not detected by the real-time PCR assay. In contrast, using the nested real-time PCR assay, the virus was detected at 10^4 $TCID_{50}$ and 10^3 $TCID_{50}$ at 10.95Ct and 21.24Ct respectively. The dilution series was not well distributed over the cycling program, and the curves amplified at erratic points. Both assays were repeated several times, but yielded similar results.

The real-time PCR assay for RSV detected the virus with a titre of 10^4 $TCID_{50}$ at 25.64 Ct, with an endpoint of 10 $TCID_{50}$, detected at 36.06 Ct (see figure 3-3).

Table 3-2 This table shows the average Ct values for each virus at various dilutions tested. The darkened cells indicate that the particular dilution was not tested for that virus. Results for both the real-time PCR assays and the nested real-time PCR assays are compared. The assay for RSV can be considered 1000 times more sensitive than represented in this table, as the first round PCR products had to be diluted 1 in 1000 before the second round of amplification, due to excess of product.

Virus		**Virus titre ($TCID_{50}$)**						
		10^5	**10^4**	**10^3**	**10^2**	**10**	**1**	**0.1**
Influenza A H1N1	Real-time		22.17 Ct	26.15 Ct	28.79 Ct	33.59 Ct	36.99 Ct	ND
	Nested		5.47 Ct	8.28 Ct	12.03 Ct	16.37 Ct	20.64 Ct	25.04 Ct
Influenza A H3N2	Real-time	25.80 Ct	27.57 Ct	30.97 Ct	34.86 Ct	ND	ND	
	Nested	5.92 Ct	8.57 Ct	11.77 Ct	15.16 Ct	17.88 Ct	20.45 Ct	
Influenza B	Real-time		ND	ND	ND	ND	ND	ND
	Nested		10.95 Ct	21.24 Ct	ND	ND	ND	ND
Parainfluenza 1	Real-time		22.28 Ct	26.06 Ct	29.14 Ct	37.06 Ct	ND	ND
	Nested		5.63 Ct	9.99 Ct	13.29 Ct	21.78 Ct	30.46 Ct	33.20 Ct
RSV	Real-time		25.64 Ct	28.92 Ct	32.63 Ct	36.06 Ct	ND	ND
	Nested		15.97 Ct	19.79 Ct	23.39 Ct	28.37 Ct	31.22 Ct	ND
Virus titre (PFU)			**10^4**	**10^3**	**10^2**	**10**	**1**	**0.1**
MS2	Real-time		23.11 Ct	26.33 Ct	30.63 Ct	33.50 Ct	ND	ND
	Nested		7.42 Ct	9.27 Ct	12.39 Ct	16.02 Ct	19.18 Ct	19.31 Ct

The nested real-time PCR assay detected the 10^4 $TCID_{50}$ titre of virus at 15.97 Ct, nearly 10 cycles earlier than the real-time PCR assay. Moreover, the nested real-time PCR assay had an endpoint of 1 $TCID_{50}$, amplifying at 31.22 Ct. The PCR products had to be diluted 1:1000, due to an excess of product resulting from the first round PCR, which exhausted the reagents and prevented the reaction from proceeding after about 8 cycles. An excellent distribution for a 10-fold dilution series for this virus was displayed by this assay, with distribution intervals between 2.85 cycles and 4.98 cycles. The virus were detected at an

average of 8.93 cycles earlier by the nested real-time PCR assay than the real-time PCR assay, after the PCR products were diluted 1:1000.

The real-time PCR assay for parainfluenza virus 1 (PIV1) had an endpoint of 10 $TCID_{50}$, which was detected at 37.06 Ct. The nested real-time PCR assay had an endpoint of 0.1 $TCID_{50}$, detected at 33.20 Ct. The amplification of the highest titre of PIV1 using the nested real-time PCR was very similar to that of the H1N1 results, with the dilution series being detected from 5.63 Ct (10^4 $TCID_{50}$). The intervals between the 10-fold dilutions ranged from 2.74 to 4.36 for the first three dilutions, and 8.49 to 8.68 cycles for the lower dilutions. The nested real-time PCR assay for PIV1 detected the dilutions at an average of about 16 cycles earlier than the real-time PCR assay.

For MS2, the real-time PCR assay, as described by O'Connell et al (2006), had an endpoint of 10 PFU, detected at 33.50 Ct. In contrast, the nested real-time PCR assay was able to detect as low as 0.1 PFU at 19.31 cycles. Furthermore, the nested assay was also able to detect MS2 at much earlier cycles in comparison with the non-nested assay. Whilst the highest dilution (10^4 PFU) was detected by the real-time PCR assay at 23.11 Ct, it was detected by the nested real-time PCR assay at 7.42 Cts. The nested real-time PCR assay for MS2 improved the detection of the virus dilutions by an average of 17.11 cycles in comparison to the real-time PCR assay. The distribution of the series, using the nested real-time PCR assay, was good and intervals were from 1.85 cycles to 3.63 cycles.

3.4.2 Application to Air Samples

Both the real-time PCR assay and the nested real-time PCR assay were used to detect MS2 from a selection of air samples in order to determine whether one had an advantage over the other in aerosol testing. Whilst the real-time PCR assay was only able to detect the virus from one air sample, the nested real-time PCR assay amplified MS2 from 14 out of the 16 samples, as seen in figure 3-4. All amplified samples were detected from 8.65 to 37.73 Cts.

There were four samples in each of the four groups, with each group of samples from different experiments in which increasing amounts of MS2 bacteriophage were aerosolised. Thus, it was expected that for each subsequent group of samples, more viruses would be present in those samples. This was observed in the results for both plaque assay and PCR.

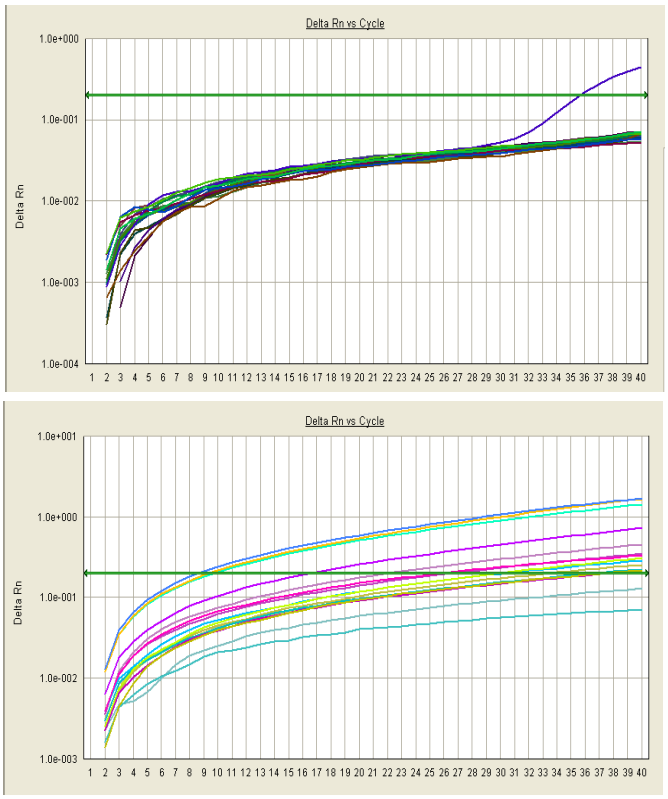


Figure 3-4. This figure shows the comparison of the TaqMan® assay (top) and the nested TaqMan® assay (bottom) when applied to air samples containing aerosolised MS2 bacteriophage.

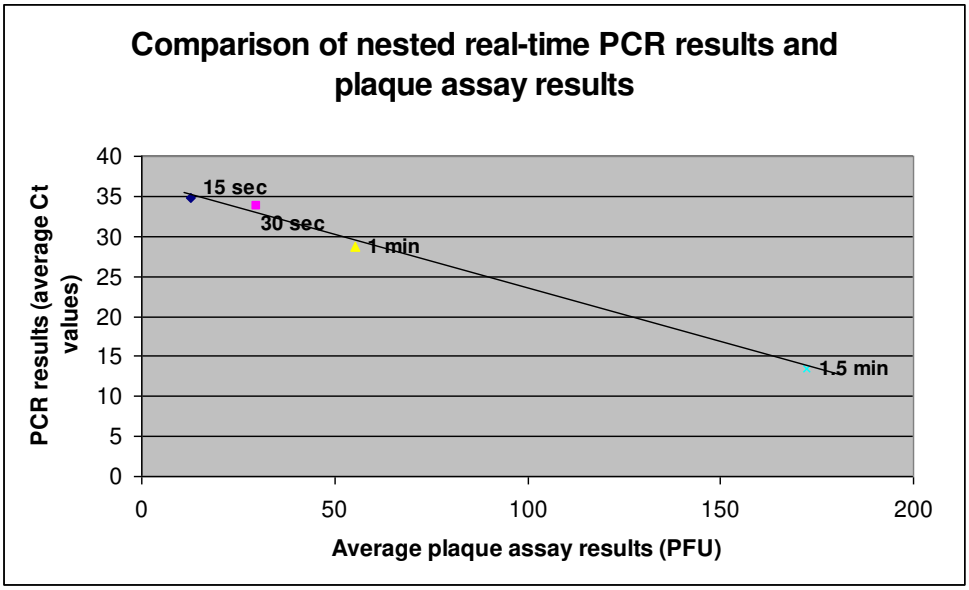


Figure 3-5 This graph demonstrates the correlation between plaque assay results (given in PFU) and real-time PCR results (average Ct values). The PCR results are given in Ct values, where a larger value indicates a smaller amount of PCR product. The higher the Ct value, the lower the number of PFU. The samples represented in this graph are from simulated aerosol experiments, in which MS2 was aerosolised from a liquid suspension for different lengths of time (15sec, 30 sec, 1 min and 1.5min).

The first group of samples, group 1, were collected during the experiment with the least amount of aerosolised MS2, by using an aerosolisation length of 15 seconds. Using the nested real-time PCR, two samples were detected at 22.03 Ct and 37.11 Ct. The other two samples were not detected. Plaque results collected from the same experiment had an average of 12.75 PFU.

The samples from group two, in which MS2 was aerosolised for 30 seconds, were all detected by the nested real-time PCR, and were detected between 26.62 Ct and 37.73 Ct, with an average of 34.79 Ct. The plaque results from group 2 had an average count of 29.75 PFU.

All of the group 3 samples, from a 1 minute aerosolisation of MS2, were also detected. The average Ct value for this group rose to 22.06 Ct, with individual results ranging between 16.57 Ct and 37.37 Ct. Similarly, the plaque results also increased from the last group, with an average of 55.25 PFU.

The final group, with a 1.5 minute aerosolisation length, had the highest plaque counts and PCR results. The nested real-time PCR detected the four samples over a range of 8.65 Ct to 26.01 Ct, with an average of 13.46 Ct, whilst the average plaque count was 172.25 PFU.

For each group, the nested real-time results showed a relative change which followed the plaque assay results (for infectious virus recovery). The results for each test are reported here as an average of the four samples. The results are shown in figure 3-5, and are compared to the recovery of infectious virus in the concurrent tests, where a linear relation between infectious virus and PCR detection point is observed.

3.5 Discussion and Conclusions

A series of nested real-time PCR assays for detection of respiratory viruses in aerosols has been successfully developed and adapted, with the advantages of increased sensitivity and lower detection limits. These are imperative features for the application of aerosol science, particularly studies involving viruses, given that they are likely to be present in air samples in low numbers and are prone to injury by the aerosolisation process, rendering viruses non-infective and thus unable to be cultured. The successful application in this study of the nested real-time PCR assay for MS2 to air samples demonstrates its superior ability to detect viruses in air samples, where the respective real-time PCR assay failed to give positive results. Additionally, in the validation tests for each assay, in each case a

substantial shift of the amplification curves compared to earlier cycles in the program was observed.

The increase in sensitivity of each nested real-time PCR assay was a 10-100 fold improvement on their respective real-time PCR assays. For this application, it is imperative that as much of the virus is detected as possible. Detection limits down to 0.1 $TCID_{50}$ or 0.1 PFU are important results, as not only do they show a marked increase in sensitivity, but also demonstrate the presence of viruses in the samples which plaque assays would not detect. In addition, the nested real-time PCR assays detected the samples at much earlier stages in the program, up to 19.5 cycles in the case of influenza A (H3N2). This shift was particularly evident in the case of RSV, where the PCR products had to be diluted to 1:1000 as the abundance of template exhausted the reagents in the second step of the real-time PCR assay. Even after the dilution of the PCR products, the curves of the dilution series still exhibited a shift of 8.93 Cts. This is important for the intended application of the assays, because it is expected that most of the samples will have low amounts of virus, and thus high sensitivity in the nested real-time PCR assay is imperative. Earlier detection in the program is indicative of a strong result, which is favourable, due to the nature of the experiment where contamination may be an issue.

Successful application of the nested real-time PCR assay to the aerosol samples where the real-time PCR assay failed is a significant result. Individually the plaque results and the PCR results do not show a large correlation; however this is due to the fact that they were samples taken from a chamber, and thus each sample will be different. However, when the samples are compiled and averaged to create a larger sample, the relationship between the plaque results and the nested real-time PCR appears. In future studies, a standard curve will be used to compare the air sample results to, in order to determine an approximate number of viruses in the samples.

One of the important benefits of using PCR in aerosol studies is that it avoids the problem of a loss of virus infectivity, as it is independent of culture (Peccia and Hernandez, 2006). This can be due to irreparable damage to viral RNA, inhibiting amplification of the viruses; long sampling times and subsequent decay in infectivity; distribution of viruses; or merely small sample sizes the can contribute to the low numbers of viruses in air samples. The first documented and successful attempt to detect rhinovirus which was aerosolised in a small chamber and captured on Teflon membranes, used semi-nested RT-PCR and reported a detection limit of 1.3 $TCID_{50}$ (Myatt *et al.*, 2003). However, using spiking experiments, the detection limit was 0.8 $TCID_{50}$, demonstrating a small loss of virus in the aerosolisation or

collection process. Given that this is a major factor in aerosol studies, PCR has a major advantage in that it does not rely on infectivity. Additionally, it has the potential to provide much quantitative information on viruses in aerosols, which was less feasible and less accurate before the advent of quantitative PCR, due to problems associated with culture techniques.

In conclusion, several new and highly sensitive PCR assays have been designed for use in aerosol studies involving respiratory viruses and a bacteriophage model (MS2), previously used in simulated experiments. The nested real-time PCR assays were more sensitive than their corresponding real-time PCR assays, detecting smaller amounts of virus at early Ct values. Additionally, both PCR assays were applied to samples taken from an aerosol experiment using MS2, with the nested real-time PCR much more successful in detecting MS2 in the samples than the respective real-time PCR assay.

In future research, this PCR method can be applied to aerosol studies using bacteriophage and as well as respiratory viruses. The successful application of the PCR to air samples will allow the technique to be used in a range of aerosol studies, including a human respiratory emission study involving subjects with influenza and other respiratory illnesses.

Chapter 4: Fate of intact MS2 bacteriophage in aerosols, produced from two different suspensions

4.1 Abstract

This Chapter describes the investigation of the physical characteristics of four different droplet types, as determined using an ultra-violet aerodynamic particle sizer. It was found that the droplets were different in size; however the main difference was in the numbers of droplets produced. Far more droplets were produced from the PBS suspension than from the artificial mucous suspensions. We also investigated the differences in the recovery rates of infectious MS2 particles from each of the four types of droplets. We found that the type of droplet did affect the ability of the bacteriophage to remain infectious in the droplet. Artificial mucous was shown to have considerably favoured the recovery of MS2 in comparison with the PBS suspension. We also found that the infectious MS2 recovered were generally recovered from larger droplets than expected based on the sizing distributions provided by the UV-APS.

4.2 Introduction

The production of droplets and aerosols is a complex process in the body, with many factors influencing the properties of the resulting particles. A common feature of previous studies is the use of a liquid nebulising suspension, such as diluent or buffered peptone water. However this does not truly represent a natural system as it fails to recognize a potentially important factor, that of mucous viscosity. Although it has been found that droplets of pure water and droplets expelled from respiratory secretions (of healthy individuals) have very similar dynamics in terms of evaporation (Morawska *et al.*, 2008), this does not necessarily mean that the recovery of intact viruses within those two types of droplets will also be similar. The exact droplet creation methods are not often fully disclosed in papers simulating this process, but it is highly probable that this question has not been addressed. In this research, the differences in virus integrity between a liquid nebulising suspension and an artificial mucous suspension as vehicles for virus transmission are explored and compared.

Studies which have been conducted to characterise the distribution of virus-containing droplets have often used models to simulate natural aerosolisation, due to the difficulties of "culturing" a virus that is pathogenic to humans. This is the case in this study, where bacteriophage MS2 was used as a model for influenza virus to permit exploration of the factors that may influence the way the viruses behave in the aerosols produced.

The main aim of this Chapter was to compare different types of droplets – 'wet' and 'dry'. This was performed for both a liquid suspension, phosphate buffered saline (PBS), and the artificial mucous nebulising suspension - thus, we examined four different types of droplet. The recovery of aerosolised viruses was measured and compared for these four types of droplets. Only "culture" techniques were used to detect viruses in this study. This was necessary to develop a better detection method, which would give a more accurate description of the presence of viruses in aerosols, to provide a comparison against which to assess a new method (applied in subsequent studies). Secondary aims of this study were: to compare the size distributions and concentrations (number of droplets per cm^3) of each of the types of droplets produced by the Collison; to examine the size distribution of virus-laden droplets; and to compare the infectivity of MS2 in the different nebulising suspensions over time at different temperatures.

4.3 Methods

Aliquots of MS2 bacteriophage and its host *E. coli,* propagated and stored as described in Section 2.1.2, were used for all aerosolisation experiments discussed in this Chapter. Collection plates containing an *E. coli* host overlay were prepared as described in Section 2.1.1 (henceforth referred to as host overlay plates). Plates were prepared on the day of the experiment for which they were to be used, and experiments were started immediately after the plates were ready.

Aerosol delivery methods were described in Section 2.2.2. Aerosol collection was performed using the six stage impactor (described in Section 2.2.3). An *E. coli* host overlay plate was placed into each of the six stages of the impactor and each experiment was performed in triplicate, thus each experiment yielded 18 plates. Plaques were counted the following day, and the positive-hole correction method (Macher, 1989) was applied. Average counts were then calculated for each experiment (per stage and total count).

For this stage of the project we held constant the following factors: concentration of virus in the nebulising suspension (10^5 PFU/mL); elapsed time between aerosolisation and sampling (zero); airflow rate through the Collison (six L/min); and aerosolisation time, which was 30 seconds for each experiment. The factors to be varied in this case were the nebulising matrix (PBS or artificial mucous – see Section 2.2.4) and the state of "moistness" of the droplets (wet or dry) as described in Section 2.2.2.

The droplets were characterised using an ultra-violet aerodynamic particle system (UV-APS; TSI Inc., Shoreview, USA) which measures particles in the range of 0.5 – 20 µm diameter. Measurements were taken to characterise the size distribution of the droplets and the number of the droplets, as produced from both the artificial mucous suspension and from a liquid suspension of phosphate buffered saline (PBS) for both wet and dry droplets. Viruses were not included in the suspensions for this part of the study, but other parameters were kept the same so as to accurately recreate the experimental conditions. This work was carried out at the International Laboratory for Air Quality and Health (ILAQH), at QUT.

Finally, in a static, non-aerosolising experiment, the recovery of MS2 was tested in PBS and artificial mucous, over a period of time (zero to 60 minutes) and against temperature (room temperature, measured as 23 °C; and on ice, zero °C). Recovery of MS2 was measured using the same host overlay method used to determine bacteriophage titre, as described in Section 2.1.2. MS2 infectivity over time was also tested in TBSA broth (see Section 2.1.2), as a basis of comparison, as one would expect retention of virus infectivity to be relatively favourable in this suspension.

A Chi-square test for one categorical variable was used to determine whether the size distribution of the virus-laden droplets ('observed' data) differed from the size distribution of droplets as generated by the Collison ('expected' data). Test statistics were obtained and probabilities were calculated using a Chi-squared table (Fawcett and Kent), with five degrees of freedom. Two-way repeated (within subjects) ANOVA was used to compare the recovery of MS2 as detected in the static experiment, to compare the effects of the suspensions, time and temperature on virus infectivity. One-way ANOVA was used to compare the mean recoveries of each of the droplet types.

Table 4-1 Size distributions and droplet concentration of the four types of droplets used in this study, as measured using the UV-APS ('PBS' – phosphate buffered saline; 'AM' - artificial mucous)

Sample	Median (µm)	Aerodynamic mean (µm)	Geometric mean (µm)	Mode (µm)	Concentration of droplets/cm^3
PBS wet	1.100	1.226	1.166	0.980	15,000
PBS dry	1.023	1.087	1.057	0.965	2303
AM wet	0.915	0.976	0.935	0.910	1842
AM dry	0.850	0.884	0.860	0.835	412

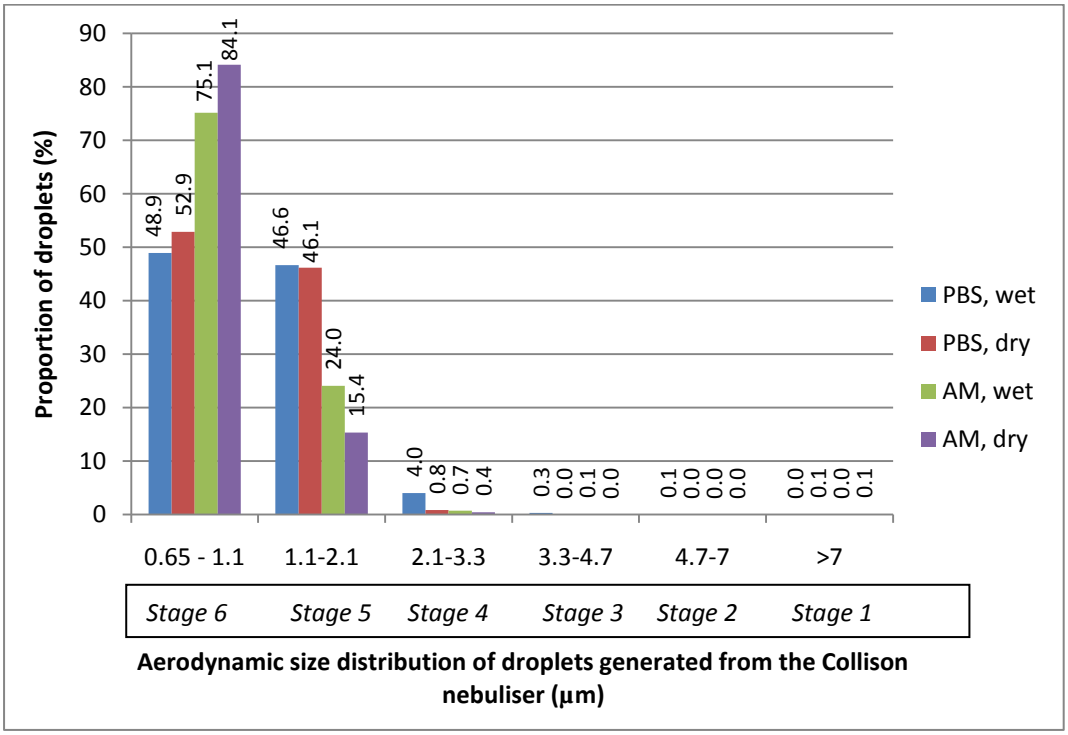


Figure 4-1 The size distribution of the four types of droplets created by the Collison and measured by the UV-APS, divided into the same size fractions as the Andersen sampler

4.4 Results

4.4.1 Droplet sizes and concentrations as measured with the UV-APS

The UV-APS showed that the majority of droplets produced by the six-jet Collison nebuliser, when operating at six L/min, ranged in aerodynamic diameter from less than 0.523µm to 2.1 µm. The four types of droplets created by the Collison (wet and dry droplets from the artificial mucous suspension and the PBS suspension) differed slightly in size (see table 4-1). The droplets with the largest diameter were the wet droplets generated from PBS, with a mode of 0.98 µm. In comparison, the mode of the dry droplets from the same suspension was 0.965 µm. The artificial mucous droplets were smaller again: wet droplets from this suspension had a mode 0.910 µm; dry droplets had a mean diameter of 0.835 µm.

The number of droplets was considerably different. There was a much higher concentration of droplets created from the PBS suspension, especially when wet droplets were aerosolised from the PBS suspension. A concentration of 15,000 droplets per cm^3 was measured using the UV-APS, whereas when wet droplets were aerosolised from the artificial mucous suspension, a concentration of only 1842 droplets per cm^3 was recorded. When dry droplets were aerosolised from the PBS suspension, the average number of droplets per cm^3 was 2303; from the artificial mucous suspension, an average of 412 droplets per cm^3 was recorded.

The size distributions of the droplets, when divided into the fractions of the Andersen six-stage sampler, are shown in figure 4-1. It showed that the majority of droplets from the artificial mucous suspension fell within the range of the last stage of the sampler; that is, the stage collecting the smallest droplets of 0.65 – 1.1 µm diameter. From this suspension, 75.1% of wet droplets and 84.1% of dry droplets fell within this size range.

Droplets from the PBS suspension were relatively evenly spread over the fifth and sixth stages of the sampler, with the majority of the droplets falling within the range of 0.65 – 1.1 µm. Based on this data, when using the six stage impactor sampler, 46.4% of wet droplets and 46.1% of dry droplets should be collected on the fifth stage of the sampler, and 48.9% of wet droplets and 52.9% of dry droplets should be collected on the sixth stage.

4.4.2 Size distribution of virus-laden aerosols

The majority of the viruses recovered were from the last two stages of the Andersen sampler; that is, the stages collecting the smallest droplets, of sizes ranging from 0.65 µm to 2.1 µm. However, there was a distinct difference in the size distributions of virus-laden droplets produced from PBS and from artificial mucous (see figure 4-2). Viruses aerosolised from the PBS suspension were recovered more frequently from the fifth stage (that is, droplets of an aerodynamic size of 1.1 – 2.1 µm) than the sixth stage (droplets with an aerodynamic size of 0.65 – 1.1 µm). For wet droplets, produced from the PBS suspension, the size distribution of virus-laden droplets was more spread out across the stages with the majority (49.3 %) in the fifth stage (size range of 1.1 – 2.1 µm). Of the remaining droplets, 26.2 % were in the fourth stage (2.1 – 3.3 µm) and 17.7 % were collected in the sixth stage (0.65 – 1.1 µm).

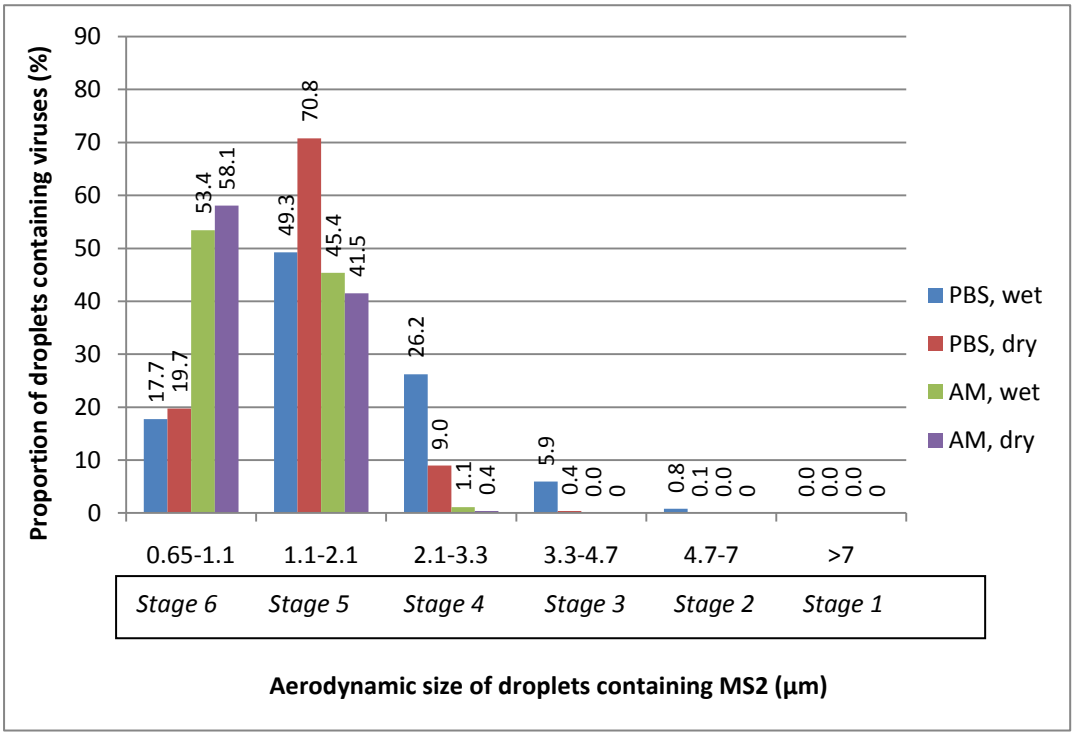


Figure 4-2 The distribution of droplet sizes that contained viruses, as collected in aerosol experiments by the six-stage Andersen sampler (PBS = phosphate buffered saline, AM = artificial mucous)

For dry droplets aerosolised from the PBS suspension, 70.8% of the viruses were within droplets in the fifth stage (size range of 1.1 – 2.1 μm); 19.7% were contained within the sixth stage (0.65 – 1.1 μm) and 9% were contained within the fourth stage (1.1 – 2.1 μm).

Droplets aerosolised from the artificial mucous suspension and containing viruses had quite a different size distribution, where the majority of droplets were collected in the last stage. For the wet droplets, 53.4% of the viruses were collected within the sixth stage, and 45.4% were collected in the fifth stage. For dry droplets, 58.1% of the viruses were contained within droplets in the size range 0.65 – 1.1 μm, and 41.5% were collected in the fifth stage. In both types of artificial mucous droplet, virtually no viruses were collected within stages one to four; that is, very few viruses were contained within droplets larger than 2.1 μm in diameter. However droplets aerosolised from PBS with an aerodynamic size >2.1 μm did contain some viruses. This effect was more pronounced in the wet droplets than the dry droplets.

Theoretically, if all droplets contained viruses, it would be expected that they should follow the same size distribution as the particle size distribution shown in Section 4.4.1. A Chi-squared test was used to assess whether this was the case: test statistics were obtained from observed and expected counts and probabilities were calculated using a Chi-squared table (Fawcett and Kent), with five degrees of freedom. We found that the size distribution of virus-laden droplets from the artificial mucous did fit with the size distribution of the total particles, as measured by the UV-APS. The results from the wet droplets had a 50% chance of fitting with the distribution of the total particles (test statistic = 3.905), whilst the dry droplets (virus-laden) had an 80% chance (test statistic = 2.447). Both types of droplets from the PBS suspension, however, did not fit with the model suggested by the total particle counts. The probabilities calculated were <0.001% for both wet and dry droplets (test statistics = 4821.234 and 5578.383 respectively).

Overall, the virus-laden droplets showed a skewing to the right, or to larger diameters, in comparison with the total particles (including droplet residues not containing viruses). This was more pronounced with the droplets from the PBS suspension than those from the artificial mucous suspension. Consequently the test statistics show that the size distributions of the virus-laden droplets are not well-fit to the total droplet measurements.

4.4.3 Effect of different suspensions and droplet types on recovery of MS2 from droplets

MS2 recovery was improved when aerosolised from an artificial mucous suspension in comparison to the droplets aerosolised from the PBS suspension. This was especially the case when the wet droplet type was produced, where an average of (3475 ± 687) PFU was recovered from a challenge of 10^5 PFU, which was a recovery rate of 3.475 % (see figure 4-3). Dry droplets produced from the artificial mucous suspension also had a relatively high recovery rate: an average of (1688 ± 313) PFU was recovered from a challenge of 10^5 PFU, a 1.688 % recovery rate.

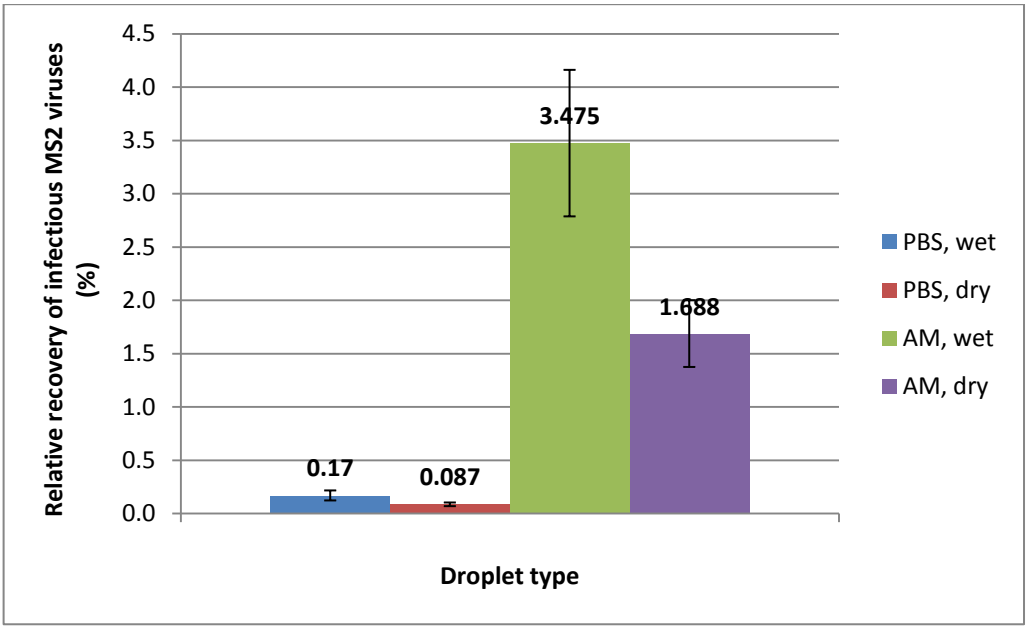


Figure 4-3 The relative recovery of viruses from challenges of 10^5 PFU in nebulising suspensions of PBS and artificial mucous, for both wet and dry droplet types

Droplets produced from PBS nebulising suspensions had much lower recoveries from the original challenge. When aerosolised within wet droplets, the average number of PFU recovered was (171 ± 47) from the 10^5 PFU challenge, equal to 0.171 %. Dry droplets yielded less PFU following aerosolisation: an average of (87.1 ± 17.1) PFU was recovered from the 10^5 PFU challenge, equal to 0.087 % of the original MS2 challenge.

The one-way ANOVA test showed that there were significant differences in MS2 infectivity between the four types of droplets. Specifically, the infectivity of viruses aerosolised in wet droplets from the artificial mucous was significantly higher than that of all other droplet

types. The largest difference was between the wet artificial mucous droplets and both wet and dry droplets from the PBS suspensions ($p = 0.001$). There was a smaller statistical difference between the recoveries from the dry artificial mucous droplets and wet droplets produced from PBS ($p = 0.084$) and dry droplets from PBS ($p = 0.067$). There was also strong evidence to suggest that the recovery of MS2 from wet and dry droplets from the artificial mucous suspension were different ($p = 0.041$). The differences between the two types of droplet from the PBS suspension was not statistically significant ($p = 0.999$).

4.4.4 Effect of temperature on MS2 recovery from various suspensions over time (non-aerosolised)

Three suspensions were tested: PBS and artificial mucous, as used in aerosolisation experiments, and the broth TBSA was used as a comparison. For each of the three suspensions, MS2 infectivity remained higher when held at room temperature (23 °C) than it did on ice. Additionally, MS2 was found to respond quite differently when held in an artificial mucous suspension at different temperatures. Whilst the levels of culturable virus dropped to 78.85 % after one hour at room temperature, when held on ice, the levels dropped to 27.90 % (see figures 4-4 and 4-5). For both temperatures, the biggest drop occurred within the first 20 minutes: at room temperature, the number of viruses dropped by 11.50 %; and on ice, the levels dropped by 69.10 %. The next time point, 20 minutes later (40 minutes from start of the experiment), saw a much smaller decrease, particularly in the ice samples, where the levels dropped to 40.53 % of the initial virus numbers. For the viruses at room temperature, the numbers decreased to 79.85 % at the 40 minute time point. Finally, the decrease in viruses for the last time point, a further 20 minutes later (60 minutes from start of the experiment), was to 78.85 % at room temperature and to 37.28 % on ice.

In comparison with the artificial mucous suspension, viruses in the TBSA suspensions showed a somewhat more linear decay pattern. Additionally, the difference between the two temperatures for MS2 infectivity when held in TBSA was not as pronounced, and temperature did not seem to have as much of an effect in comparison to what was observed in the artificial mucous samples. For MS2 held in TBSA at room temperature, the levels of infectious MS2 dropped to 94.05 % after 20 minutes, 85.85 % after 40 minutes, and 66.85 % after 60 minutes. When held on ice (0 °C) in TBSA, the levels dropped to 91.93 % after 20 minutes, 79.82 % after 40 minutes, and 63.423% after 60 minutes.

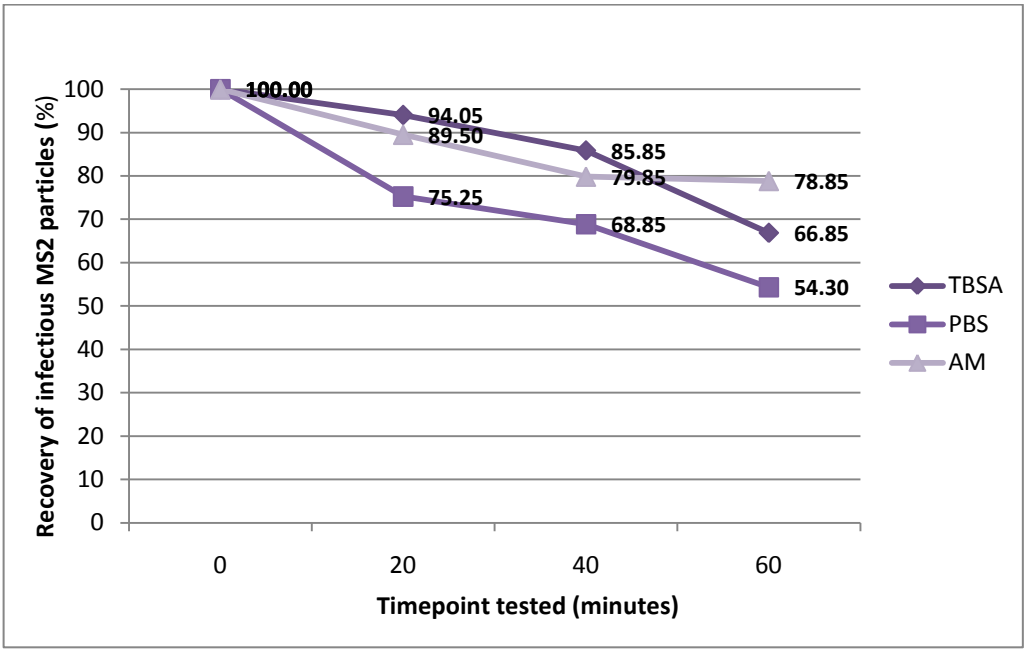


Figure 4-4 MS2 bacteriophage decay over time in various suspensions, at room temperature (23 °C)

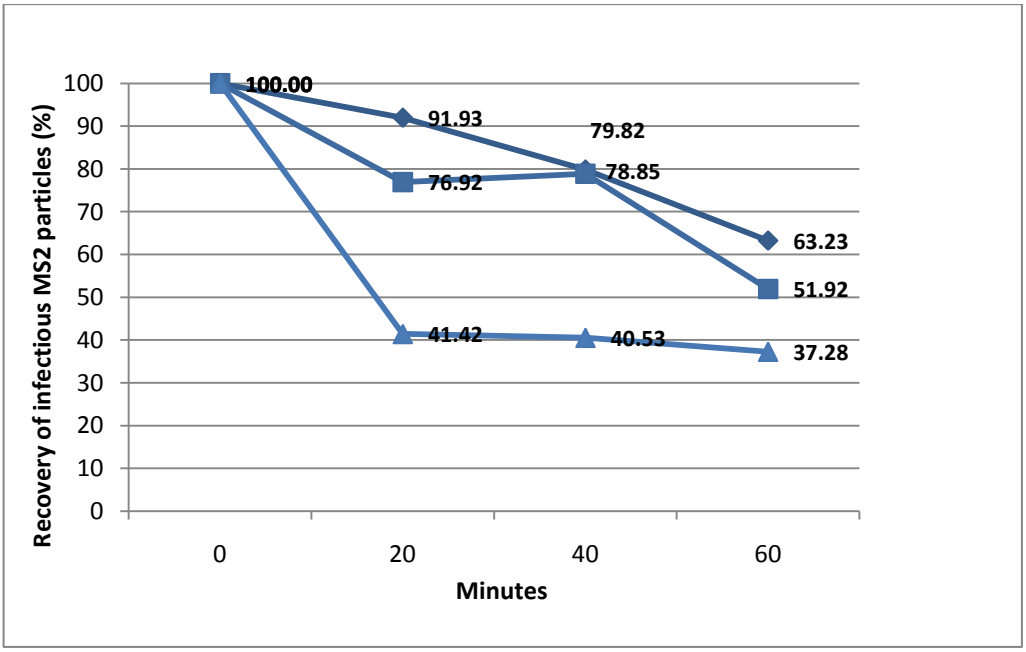


Figure 4-5 MS2 bacteriophage decay over time in various suspensions, on ice (0 °C)

When held in the PBS suspension, the levels of infectious MS2 were more similar to the levels measured when it was held in TBSA, than those of the artificial mucus. After 20 minutes held at room temperature, the levels decreased to 75.25 %, then decreased to 68.85 % after 40 minutes and 54.30 % after 60 minutes. However, when held on ice, the levels of infectious MS2 dropped to 76.92 % after 20 minutes, 78.85 % after 40 minutes and 51.92 % after 60 minutes. The slight discrepancy occurring between the 20 minute and 40 minute time point may be due to clumping of virus particles in the suspension, or may be due to a pipetting error.

The two-way repeated (within subjects) ANOVA test showed that infectivity of MS2 was significantly reduced over time ($p < 0.001$). There was strong evidence that reduction of virus infectivity was significantly different when held at different temperatures ($p = 0.022$). Furthermore, MS2 infectivity was significantly decreased over time when held in different suspensions ($p = 0.004$). Specifically, there was reasonably strong evidence that the TBSA suspensions significantly favoured virus infectivity over the artificial mucous suspensions ($p = 0.035$). The joint effects of all variables had a significant effect on the infectivity of MS2 ($p = 0.004$).

4.5 Discussion and Conclusions

The differences between the PBS nebulising suspension and the artificial mucous suspension were very interesting. It was clear that the artificial mucous suspension had a protective effect on the viruses on immediate release. More than three times the amount of viruses remained infectious when aerosolised from the artificial mucous suspension compared to droplets produced from the PBS suspension. It may follow that the physical properties of human mucous may have a protective effect on the viruses. However, when examining the results from the assay assessing infectivity at different time points and temperatures, it would seem that MS2 viruses do not remain infectious for long when held in artificial mucous on ice. This may have had an effect on the results, as the nebulising suspensions were kept on ice during experiments and between replicates, on the assumption that it would slow the decay of the suspended viruses. This assumption is quite generally held by researchers in this field, and is described in the literature (Olson *et al.*, 2004). If this is the case, it is possible that the recovery of viruses may be improved when aerosolised from the artificial mucous suspension, had they not been stored on ice between experiments and replicates. As mentioned, this is a common practice, and it may

be inferred that modelling of virus recoveries in historical research has consistently under-estimated the probable recovery in real-life.

It was interesting to observe differences in droplets produced from the different types of nebulising suspension. The droplet sizes all differed slightly, but it was the concentration of the droplets which yielded the most unexpected results. The physical process of creating droplets through atomisation (i.e. with the Collison) influences the resulting droplets; this is governed by surface tension and viscosity vs. aerodynamic forces (Morawska, 2006). This was evident in the differences observed in droplets produced from the two types of nebulising matrix, PBS and artificial mucous, which had differing viscosities, and thus, surface tensions. Considering that the concentration of wet droplets (per cm^3) produced from the PBS suspension was approximately eight times that of the wet droplets from the artificial mucous suspension, this gives strong support to the theory that artificial mucous imparts a protective effect on virus infectivity, as the MS2 recovery from artificial mucous was 20 times that from PBS droplets. A possible future direction of this research would be to use a different nebulisation approach that would allow the production of a higher concentration of droplets from the artificial mucous suspensions. This would allow further exploration of the fate of viruses in aerosols.

Observation of the droplet size data in figures 4-1 and 4-2 reveals that the droplets resulting from the Collison operating at 6 L/min, including both virus-laden and non-virus-laden droplets, mostly fall in the size range of 0.65-2.1 µm for all four types of droplets. Proportionally, the dry droplets were smaller in aerodynamic diameter than their wet droplet counterparts, no doubt due to further evaporation of the dry droplets than the wet droplets. Given that the Collison is reported to produce 2 µm droplets when operated at 6 L/min, and that droplets usually evaporate to around half their size, these findings fall into the expected size range. The shift in particle sizes as seen in figure 4-2 can be attributed to the fact that the observed droplets are virus-laden, rather than droplet residues, which are measured by the UV-APS and included in figure 4-1. This explains why there is a higher proportion of smaller droplets evident in this figure. It could also be inferred that evaporation of droplets containing viruses is less pronounced than those which do not contain viruses, due to the droplet composition (less water content is a possibility).

The chemical properties of human mucous, and furthermore mucous of an infected person (which may be altered chemically, immunologically and physically in a disease state) have not been explored here, and could exert another influence over virus infectivity altogether.

Without data from human studies, this conjecture can be neither confirmed nor denied; in fact there is very little information to be found on the chemical properties of a human's respiratory secretions and mucous. However it was not within the scope of this study to investigate the chemical or biological properties of human mucous, only to assess the physical effect a mucous-like matrix may have on virus infectivity following aerosolisation. It would be a logical further step in this field of research to take chemical and biological properties into account.

The fact that 0.087 – 3.475 % of viruses remains infectious (that is, able to form a plaque) can be directly related to the aerosolisation and collection processes. Both exert some stress to the viruses, as the process incorporates shearing during the aerosolisation process and impaction onto a solid surface. In laboratory experiments, the aerosolisation process using the Collison six-jet nebuliser exerts enormous pressure and force on the virus suspension, which is forced out of the jets at six litres per minute. This is followed by a sometimes lengthy collection process; in the case of impaction, the viruses are drawn into the sampler by vacuum at 28.3 L/min, and collide with the surface of the collection medium. So it is not surprising that the relative recoveries observed in these set of experiments did not exceed 3.5 %. These results are still quite high in comparison to previous studies: one study reported a 'microbial kill' of 99.8 % when examining aerosolisation of rhinoviruses, a recovery of 0.2 % (Scarpino *et al.*, 1998), which is comparable with our results.

This study provides valuable insight into the behaviour of the bacteriophage and the effects of the different droplet types on their fate, which may inform studies of human respiratory viruses. Subsequent examination of intact bacteriophage fate, as determined by physical factors, will further our understanding of how viruses may persist in respiratory aerosols.

Chapter 5: Effect of virus concentration in the nebulising suspension and effect of elapsed time between aerosolisation and collection, on recovery of intact MS2 bacteriophage

5.1 Abstract

Physical parameters were varied in this Chapter to investigate the limitations of the infectivity of MS2 in suspended droplets following aerosolisation. The parameters that were varied were the concentration of the MS2 in the nebulising suspensions, and the elapsed time that the aerosols were held in the chamber before being collected. These variables were tested for each of the four types of droplet discussed in the previous Chapter, using the plaque assay. It was discovered that MS2 remains infectious in the artificial mucous droplets at lower concentrations, at much higher rates than those in droplets generated from PBS (these were collected immediately after aerosolisation). However, when collected after elapsed times of two minutes and longer, MS2 did not remain infectious in the artificial mucous droplets. They did however remain infectious in the PBS droplets, up to five minutes following aerosolisation in the wet droplets and up to (and possibly longer than) 20 minutes in the dry droplets. Plates placed on the floor of the chamber revealed that more artificial mucous droplets fell out of the chamber air onto the floor than droplets from the PBS suspensions. We also were able to show that when generating PBS wet droplets, the proportion of infectious MS2 detected on the floor of the chamber increased with increasing elapsed time, showing that droplets were falling out onto the floor of the chamber. From the dry PBS droplets, there was an initial increase in the infectious organisms detected on the floor of the chamber, but this then decreased at the last time point.

5.2 Introduction

An increasing number of studies have investigated the detection and/or fate of viruses in droplets; however extensive study of the persistence of influenza in aerosols has not been undertaken, nor has it been performed on the MS2 which is often used as a surrogate for

influenza studies. Of particular interest is the quantification of intact viruses when limiting factors are applied. We were able to locate only two reports of studies describing the quantification of influenza, as dictated by varying factors. As so few studies have been conducted regarding the actual aerosol characteristics of MS2 or influenza, it is logical to begin with simulated studies so that they can provide a basis of comparison and assist in our knowledge and application of these methods to studies involving infected individuals. Moreover, it is important to simulate these studies as closely as possible to real-life so that fewer external factors need to be taken into account.

From previous simulated droplet experiments, it is known that virus integrity and spatial distribution of droplets are dependent on a number of governing factors including aerodynamic droplet size and atmospheric conditions. Extensive studies examining the effect of temperature and relative humidity have also been conducted. Given that these factors have been described in the literature (Section 1.2.2.2) quite satisfactorily, they were not examined in this study. Instead, other limiting physical factors were investigated.

The objective of this study was to characterise the fate of MS2 in aerosols produced from the four different aerosol types, as described in Chapter four, whilst varying the following physical factors: the concentration of the MS2 in the nebulising suspension from which aerosols were produced; and the length of time that a virus might remain intact in suspended aerosols.

5.3 Methods

Aliquots of MS2 bacteriophage and its host *E. coli,* propagated and stored as described in Section 2.1.2, were used for all aerosolisation experiments discussed in this Chapter. Collection plates containing an *E. coli* host overlay were prepared as described in Section 2.1.1. Plates were prepared on the day of the experiment, and experiments commenced immediately after the plates were ready.

Aerosol delivery methods were described in Section 2.3.2. Aerosol collection was performed using the six stage impactor (described in Section 2.3.3). An *E. coli* collection plate was placed into each of the six stages and each experiment was performed in triplicate, thus each experiment yielded 18 plates. Plaques were counted the following day, and the positive-hole correction method was applied. Average (mean) counts were then calculated for each experiment.

For this stage of the project we held constant the following factors during 'standard' experiments: concentration of virus in the nebulising suspension at 10^5 PFU/mL; elapsed time between aerosolisation and sampling (zero); airflow rate through the Collison (six L/min); and aerosolisation which was performed for 30 seconds for each experiment. The factors to be varied in this study were: concentration of virus in the nebulising suspension (10^4 and 10^3 PFU/mL); and elapsed time between aerosolisation and sampling (2, 5, 10 and 20 minutes). Constants were maintained as above during investigation of each variable.

A one-way ANOVA test was used to determine whether there was a significant difference between the average recoveries of MS2 from droplets as tested by the plaque assay.

5.4 Results

5.4.1 Effect of varying MS2 concentration in the nebulising suspension on recovery of MS2 from droplets

The artificial mucous improved the recovery of MS2 in droplets at all concentrations in comparison to the PBS suspension. When aerosolised from the PBS suspension in wet droplets, an average of 171 (± 47) PFU of MS2 was recovered from a 10^5 PFU challenge, a 0.171% recovery rate. When aerosolised from the same suspension but in dry droplets, an average of 87.1 (± 17.1) PFU was recovered, which was a relative recovery of 0.087% (see figure 5-1), roughly half of the recovery achieved from wet droplets. When wet droplets were aerosolised from the PBS suspension and at an initial challenge concentration of 10^4 PFU, only 2.24 (± 1.21) PFU were recovered on average; this was a recovery rate of only 0.022%. Dry droplets from the same nebulising challenge yielded an average of 0.440 (± 0.439) PFU, corresponding to a recovery rate of 0.0006%. No viruses were recovered from a PBS nebulising suspension of 10^3 PFU/mL, from either wet or dry droplets.

A two-way ANOVA test showed that concentration of viruses in the initial nebulising suspension did significantly affect the relative recovery of viruses in droplets ($p = 0.001$). Additionally, the type of droplet had a significant effect on the proportion of viruses recovered ($p = 0.003$), and the interaction of droplet type and virus concentration in the nebulising suspension also had a significant effect on virus recovery ($p = 0.004$).

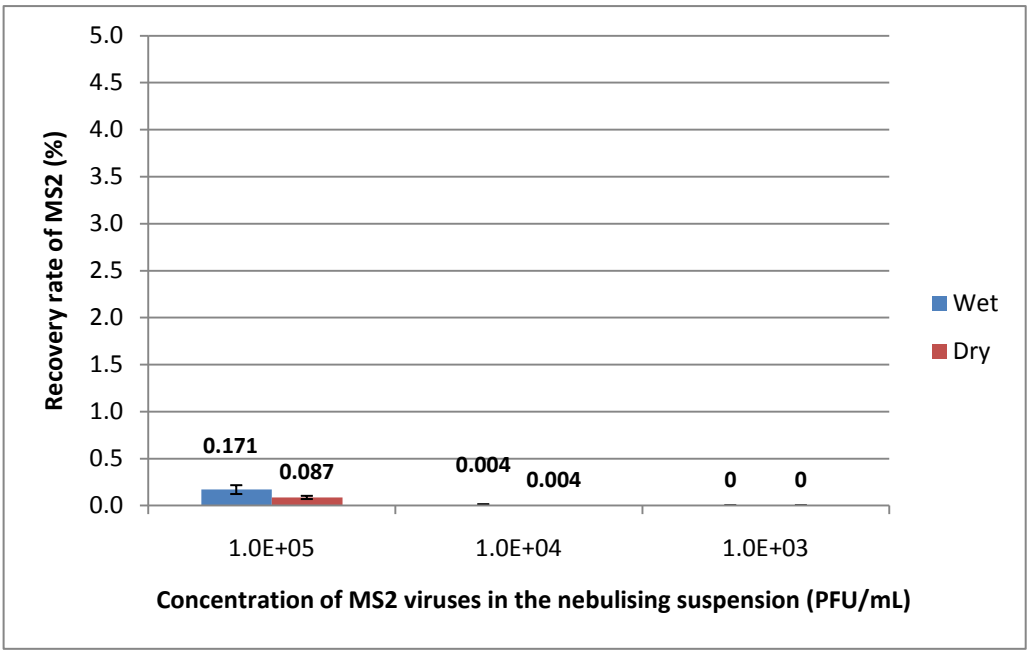


Figure 5-1 Comparison of MS2 bacteriophage recovery from droplets produced from PBS nebulising suspensions containing varying virus concentrations

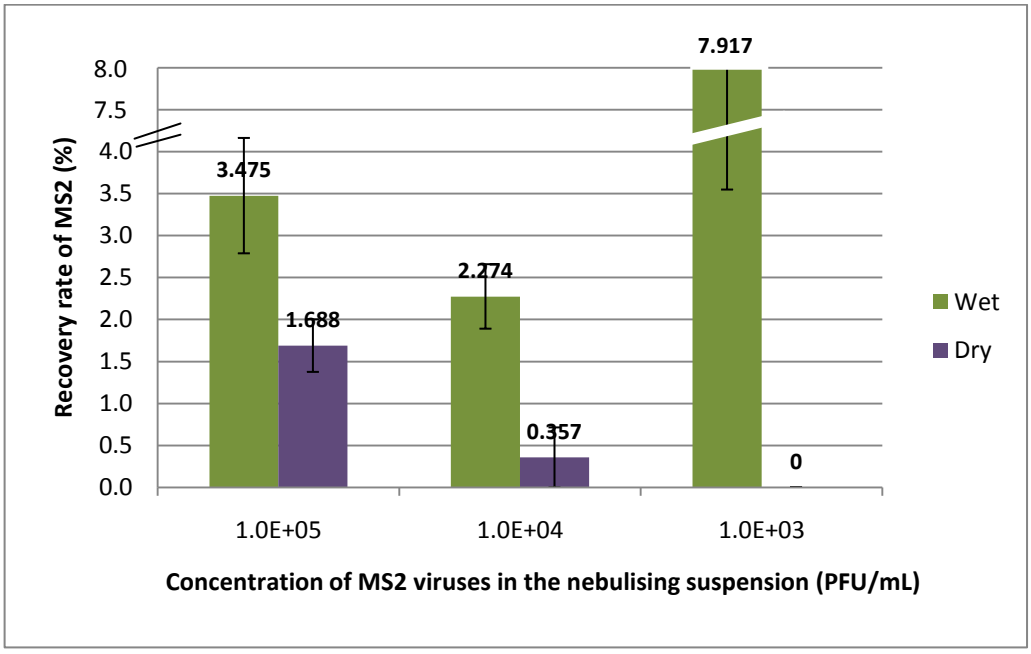


Figure 5-2 Comparison of MS2 bacteriophage recovery from droplets aerosolised from artificial mucus suspensions of varying virus concentrations (note that the concentration from wet droplets at 10^3 PFU/mL was in fact 7.9%, beyond the limits of the scale, which has been scaled with a maximum of 5% to be consistent with Figure 5-1)

In comparison, when artificial mucus was used as a suspending matrix, an average of 3474 (± 687) PFU was recovered from wet droplets aerosolised from the suspension with a challenge amount of 10^5 PFU (3.474 %). This is an increase in recovery of 20-fold (relative to that of the PBS droplets). When aerosolised in dry droplets, an average of 1688 (± 313) PFU was recovered, a rate of 1.688 % (see figure 5-2). Wet droplets produced from a challenge concentration of 10^4 PFU yielded an average of 227.4 (± 38.4) PFU, corresponding to 2.274 % recovery rate. From dry droplets of the same challenge, 35.71 (± 35.7) PFU were recovered on average (0.357 %). The recovery for viruses from a challenge of 10^3 PFU, aerosolised in wet droplets, increased to 7.917 %, with an average of 79.17 (± 43.7) PFU recovered (shown as maximum limit on the graph). No viruses were able to be recovered from this challenge concentration when aerosolised within dry droplets.

A two-way ANOVA test showed that the concentration of viruses in the artificial mucous nebulising suspensions did not have a significant impact on the recovery of the viruses ($p = 0.830$). The type of droplet also did not have a significant effect on the recovery of viruses ($p = 0.167$), and the interaction of both factors also had no significant impact ($p = 0.175$).

5.4.2 Effect of varying elapsed time in the chamber on recovery of MS2 from droplets

There was a sharp drop in the levels of culturable, or intact, viruses when examining varying lengths of elapsed time between aerosolisation and collection. This was evident in all types of droplet.

Immediately after aerosolisation (a zero minute hold before sampling), an average of 171 (± 47) PFU was recovered from wet droplets of a PBS nebulising suspension with a challenge of 10^5 PFU, 0.171 % of the original challenge. However, after only two minutes elapse between aerosolisation and sample collection, the average number of culturable viruses still suspended in the air decreased to 6.02 (± 4.5) PFU, or 0.006 % of the original challenge. This number was relatively stable until the fifth minute following aerosolisation, when an average of 4.14 (± 4.14) PFU (0.004 %) was recovered. However in between five and ten minutes following aerosolisation, the number of viruses again decreased sharply, down to an average of 0.207 (± 0.207) PFU or 0.0002 % of the original challenge. No viruses were recovered after 20 minutes.

When dry droplets were aerosolised from the PBS suspension, the recovery somewhat improved in comparison with the wet droplets. Immediately after aerosolisation, 87.1 (± 17.1) PFU were recovered on average, decreasing to 36.7 (± 14.9) PFU after two minutes of elapsed time. After five minutes, the average number of viruses dropped only slightly to 31.9 (± 30.5) PFU. However after this point, the number of viruses dropped sharply: after ten minutes elapsed they dropped to 1.44 (± 0.99) PFU and after 20 minutes elapsed they rose slightly to 11.17 (± 11.17) PFU.

A two-way ANOVA test showed that although the droplet type (wet or dry) did not have an effect on the recovery of viruses ($p = 0.842$), the time between aerosolisation and collection did have a significant effect ($p < 0.001$). There was also strong evidence to suggest that the interaction of droplet type and elapsed time did have a significant impact on virus integrity ($p = 0.052$).

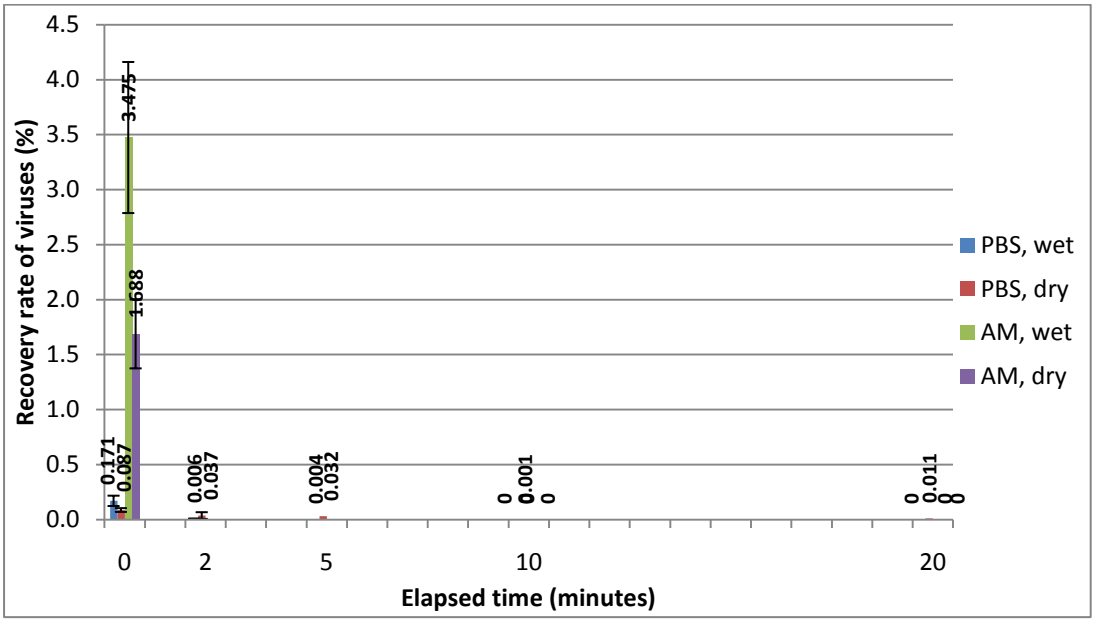


Figure 5-3 The relative recovery of MS2 from the four different droplet types. Recoveries have been normalised to an initial challenge of 10^5 PFU, collected after different elapsed times of zero minutes, two minutes, five minutes and ten minutes. (AM = artificial mucus)

Suspension in artificial mucous did not improve the recovery of the bacteriophage, held (before collection) for longer periods of time. The initial number of viruses recovered was higher than its PBS counterpart, at an average of 3,474.7 PFU (3.4747 %) from a challenge of 10^5 PFU. However no viruses were recovered at either 10 or 20 minutes after aerosolisation (see figure 5.3).

5.4.3 Surface distribution of virus-laden droplets

Viruses contained within droplets were detected on the surface collection plates in the chamber (see figure 5-4). When wet droplets were aerosolised from the PBS nebulising suspensions with a concentration of 10^6 PFU/mL, they yielded an average of 17.27 (± 17.27) PFU (0.017 %), on the floor of the chamber, within five minutes following aerosolisation. When aerosolised in dry droplets from the same suspension, only 2.31 (± 2.31) PFU were recovered (0.002 %).

The experiments showed that there was much higher fallout from the artificial mucous droplets than the PBS droplets. When aerosolised in wet droplets, an average of 526.9 (± 285.3) PFU (0.527 %) fell out onto the surface of the chamber from the 10^6 PFU artificial mucous challenge. This decreased for the dry droplets of the same suspension: an average of 368.9 (± 115.2) PFU was recovered on the surface collection plates (0.369 %). A one-way ANOVA showed that there was no statistically significant difference between MS2 recoveries from the four types of droplet ($p = 0.104$).

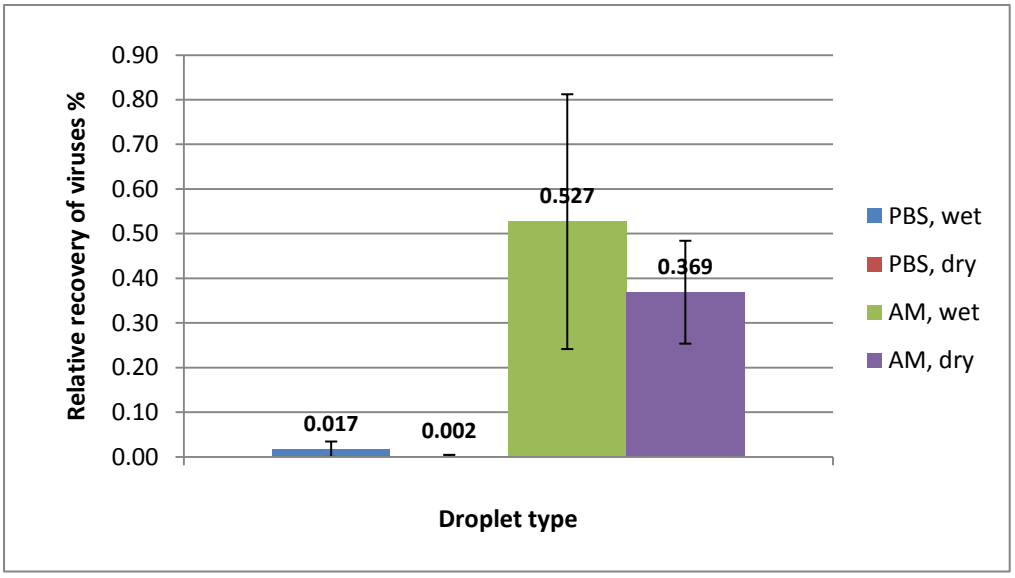


Figure 5-4 The relative recovery of viruses on the chamber floor from the original challenge of 10^6 PFU, from both a PBS nebulising suspension and an artificial mucous nebulising suspension

5.4.3.1 Surface distribution of MS2 in droplets from nebulising suspensions of varying virus concentrations

This variable was only explored for the PBS nebulising suspension, as part of preliminary work to gain a basic understanding of droplet fallout. When aerosolised within wet

droplets, the recovery of viruses increased from a challenge of 10^5 PFU (see figure 5-5). However, nothing was recovered from a nebulising suspension with a concentration of 10^4 PFU/mL. No viruses were detected on the surface collection plates when aerosolised in dry droplets, from challenge concentrations of either 10^5 or 10^4 PFU.

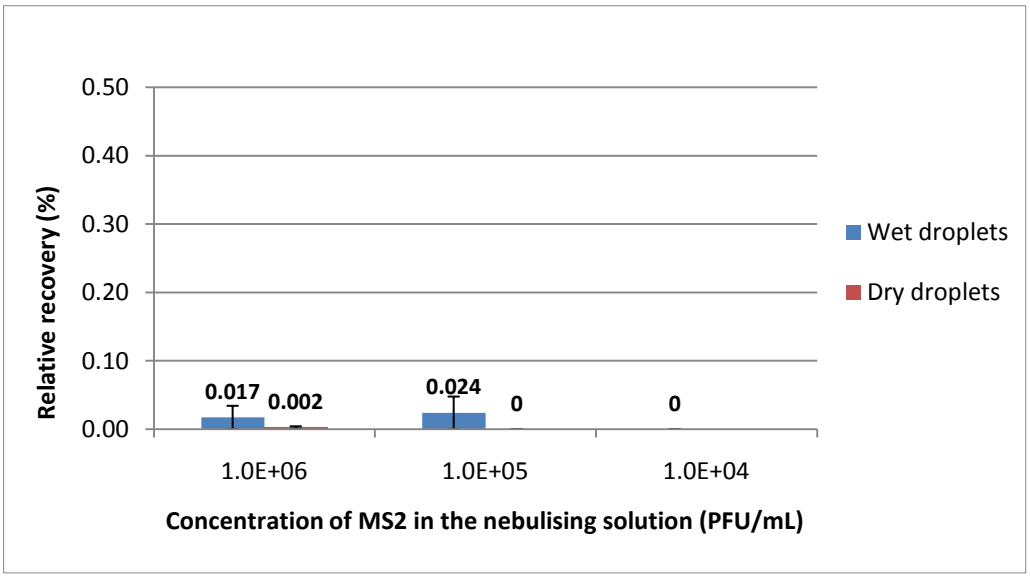


Figure 5-5 Recovery of viruses from the floor of the chamber following aerosolisation from PBS nebulising suspensions of varying concentrations

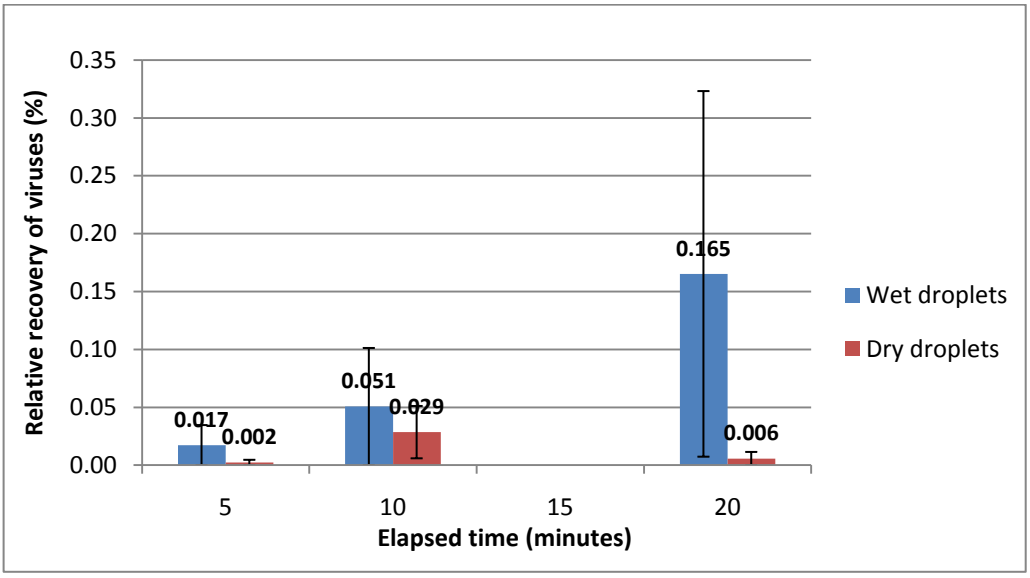


Figure 5-6 Recovery of viruses from the floor of the chamber following aerosolisation from PBS suspensions, collected at different elapsed times

5.4.3.2 Surface distribution of viruses collected after varying elapsed times

The time variable was only examined for the PBS nebulising suspension as part of some preliminary experiments. When investigating time as a factor in droplet fall-out, it appeared that the numbers of viruses did increase significantly over time, when using PBS as the nebulising suspension (see figure 5-6). When aerosolised in wet droplets, 0.017 % of viruses from the original challenge were collected onto the settle plates after a five minute elapse. This increased to 0.051 % after 10 minutes and 0.165 % after 20 minutes. A one-way ANOVA test showed that the change in recovery of viruses from the chamber floor was not statistically significant ($p = 0.555$).

When aerosolised in dry droplets, 0.002% of the viruses were recovered after a five minute elapse, increasing to 0.029% after a 10 minute elapse and then decreasing again to 0.006% after a 20 minute elapse. A one-way ANOVA test showed that the change in recovery of viruses from the chamber floor was not statistically significant ($p = 0.270$).

In combining the average numbers of viruses collected from the floor of the chamber and the air in the chamber, and normalising the results to represent collection from a 10^5 PFU challenge, two different trends are evident depending on the droplet type (see table 5-1). From wet droplets, the viruses increase quite strongly on the floor of the chamber, and decrease slightly in the air: the overall effect shows the total virus count dipped between five and ten minutes elapsed time, but after 20 minutes, the same number of viruses, as found at the immediate collection is evident.

For the dry droplets, the levels of viruses were relatively stable. An original count of 87.13 PFU drops to 34.2 PFU after five minutes, and is maintained to 29.95 PFU after ten minutes. This level then drops to 16.87 PFU after the 20 minute time point elapses.

Table 5-1 This table shows normalised data from experiments, to give an idea of the total number of viruses collected in the chamber, both in the air and on the floor of the chamber

		0 minute elapse	5 minute elapse	10 minute elapse	20 minute elapse
PBS, wet droplets	Surface	NT	17.3	50.6	165.2
	Air	171	4.1	0.2	0
	Total	**171**	**21.4**	**50.8**	**165.2**
PBS, dry droplets	Surface	NT	2.3	28.5	5.7
	Air	87.1	31.9	1.5	11.2
	Total	**87.1**	**34.2**	**30.0**	**16.8**

5.5 Discussion and Conclusions

We successfully demonstrated that, when held in a PBS nebulising suspension, MS2 bacteriophage can be detected by plaque assay (indicating intact/infectious virions) up to ten minutes following aerosolisation in wet droplets, and up to and possibly beyond 20 minutes after aerosolisation in dry droplets. When MS2 was aerosolised from the artificial mucus suspension, it was not detected by the plaque assay at ten minutes or 20 minutes following aerosolisation (from wet and dry droplets). When the concentration of MS2 was varied in the PBS nebulising suspension, the limit of detection for the plaque assay was 10^4 PFU/mL for both wet and dry droplets. When the concentration was varied within the artificial mucus suspension, the plaque assay detection limit was a nebulising suspension concentration of 10^4 PFU/mL for the dry droplets and 10^3 PFU/mL for the wet droplets.

One explanation for the almost immediate decay in MS2 infectivity when held in the artificial mucous suspension is that the suspension actually had a degrading effect on virus infectivity over time, as observed in the experiment examining this at different temperatures and times (Section 4.3.4). It showed that when MS2 was held on ice, the numbers of infective MS2 dramatically decreased: after 20 minutes, 58.58 % of the viruses were no longer able to form plaques, whereas when they were held in the TBSA and the PBS, the levels dropped by only 8.07 % and 23.08 % respectively. Given that the nebulising suspensions, within the Collison nebuliser, were kept on ice during the experiments and between replicates, this could certainly explain why the decay of MS2 infectivity was so pronounced.

Another possibility is that the protective effect of the artificial mucous was so pronounced that it actually prevented the virus particles from contacting the agar and host cells, impeding replication. This could be particularly evidenced over time, with drying of the droplet exacerbating the problem and preventing detection of the viruses using these methods. Verification of this would require a more comprehensive study of the chemical and physical properties of the artificial mucous, which lie outside the scope of this project.

Utrup and Frey found that 25 minutes following the initial aerosolisation, the levels of detectable MS2 had dropped by 28 % relative to the sample taken five minutes after aerosolisation (Utrup and Frey, 2004). This is a higher recovery than ours, however their methods and equipment differed so realistically the results cannot be directly compared.

When taking into account both the surface data and the aerosol data, the numbers of viruses aerosolised from the PBS suspensions change according to the droplet type. For wet

droplets, the numbers start off quite high at 171 PFU, then dip at the five and ten minute elapsed time points to 21.4 PFU and then 50.8 PFU; this then picks up after 20 minutes elapsed time to 165.2 PFU. This increasing number of viruses in the chamber was unexpected. One explanation for this is that the numbers of viruses suspended in droplets responsible for this actually remain infectious in the droplets for longer than is shown here, but upon impaction in the sampler, they may no longer be infectious. This would account for the increase in the numbers of viruses collected from the settle plates; they may be from droplets which were suspended in the air but fell out onto the chamber floor between 10 and 20 minutes, and remained infectious as there was no harm exerted on them in their collection.

When MS2 was aerosolised from the dry droplets, the total numbers of viruses decreased in total over the time tested. The decrease seen after five minutes elapsed time was more conserved than that of the wet droplets, and the level remained relatively stable to ten minutes before dropping slightly again at the 20 minute time point. This is a trend that might be expected, as viruses decay in the environment over time.

Chapter 6: Comparison of detection of MS2 bacteriophage using the plaque assay and the nested real-time PCR assay

6.1 Abstract

The nested real-time PCR assay, described in Chapter 3, was used in parallel with a plaque assay in order to determine whether the results would be related, and also to determine whether viral RNA was still detectable when infectious viruses were not, due to the limits of the detection method. We found that the nested real-time PCR assay was not always as consistent as the plaque assay, but was able to detect MS2 RNA copies when no infectious viruses were detected. The nested real-time PCR assay in particular, detected larger proportions of RNA copies in the droplets than infectious bacteriophages, when compared to the original amounts of bacteriophage present. However, for the droplets generated from the artificial mucous, this was not the case. RNA copies were able to be detected in all samples, but mostly at lower recovery rates than the plaque assay.

6.2 Introduction

Prior to 1995, virtually all studies that examined viral transport in aerosols used culture methods, by employing a suitable host cell layer to examine for infected regions or plaques. The number of plaques was taken to indicate the number of intact virus particles; hence the unit of PFU (plaque-forming-units) per unit volume was used. PFU has traditionally been used for such studies. However, it is now thought that only a fraction of viruses present may be capable of forming a plaque in a host layer after the aerosolisation process, due to physical damage to the virion, and thus the traditional methods very probably underestimate the actual number of infectious viruses remaining in the aerosols. In addition, there is no accurate information available as to how many intact virus particles are required for the infection of a host cell. Instinctively, the answer would be that just one virus particle per host cell was required, but this has not yet been proven conclusively.

The general lack of research in this area can be attributed to a lack of suitable virus detection methods. Traditionally, culture methods have been used to detect micro-

organisms in air samples; however, the complex nature of cell culture required for viruses presents numerous problems and no standard method has been available that integrates cell culture with an air sampling system. The shift towards the molecular detection method polymerase chain reaction, or PCR, in recent aerosol research, has presented a timely opportunity to design a method which allows collection, detection, and quantification of viruses in an air sample.

The rapid development of PCR techniques has allowed more suitable application of this technique to the problem of detecting viruses in aerosols over the past decade. This has allowed us to detect not only the "culturable" or intact virions in the aerosols, but all intact virus RNA. In developing such a method, it was important to note the likelihood of low viral concentrations in respiratory aerosols, as discussed in Chapter three.

Further to the development of the nested real-time PCR, it was necessary to develop a standard curve to quantify the collected viruses. It was decided that a synthetic RNA standard was the most appropriate option; this circumvented the problem of extraneous DNA and RNA in viral RNA extracted from the viruses, which can give a falsely increased amount of RNA. The synthetic RNA was constructed from DNA oligonucleotides and underwent several clean up steps to ensure the quality of the RNA was appropriate for PCR.

This Chapter focuses on the application of the PCR method reported in Chapter three, and the subsequent results from the aerosolisation experiments. The variables are the same as in the previous chapters (Chapters 4 and 5); only the collection method differed slightly to permit application of PCR techniques, and the detection of the viruses was based on the presence of virus RNA instead of the culturability of the collected viruses.

The objective of this study was to apply the PCR method to detect the presence of the viral RNA in aerosols, and to compare its sensitivity and accuracy for estimation of viruses through detection of RNA, with that of the traditional plaque assay described in chapters four and five.

6.3 Methods

As briefly outlined in the Section 2.2.3, a single stage BioStage® impactor sampler (SKC) was used to collect the air samples. The impactor collected all droplets larger than 0.65 μm, and was equivalent to last the stage of the Andersen six-stage impactor. Wet and dry droplets

were examined in this Chapter, from both PBS and artificial mucous suspensions. A petri dish filled with agar (non-nutritional) was prepared and a surface layer of buffer was used to collect the aerosols. The agar was necessary because the impactor samplers operate on the basis of a certain height of collecting surface, so the buffer could not simply be placed on the base of the petri-dish. A measured volume of 1.8 mL of phosphate buffered saline (PBS) buffer was used because our tests performed in developing this method showed that this was the minimum amount that would allow collection of material following sampling. As some evaporation occurred, the amount of remaining buffer that was collected from the petri dish was recorded for each experiment, measured by weight, and compared to the initial weight of buffer. The amount of buffer sample from which RNA was extracted was expressed as a dilution of the amount collected, and accounted for in the quantification stage of analysis. Experiments were performed in triplicate.

It was not feasible to use the Andersen six-stage impactor for these experiments, since the addition of buffer on the surface of the agar collection dishes made it impractical to load and use a six-stage sampler, due to a serious risk of leakage. This would not have been an issue for MS2 experiments, but would have been a major concern when flu viruses were tested. Following aerosol collection, RNA was extracted from the buffer samples as described in Section 2.2.6.

The standard experiments (that is, the experiments where variables were held constant and results were regarded as the baseline) held the following factors constant: concentration of virus in the nebulising suspension (10^5 PFU/mL); elapsed time between aerosolisation and sampling (zero); airflow rate through the Collison (six L/min); and aerosolisation which was performed for 30 seconds for each experiment. The variable factors in this study were: concentration of virus in the nebulising suspension (10^4 and 10^3 PFU/mL); and elapsed time between aerosolisation and sampling (2, 5, 10 and 20 minutes). Constants were maintained as above during investigation of each variable.

The correlation coefficients of the standard curves, used to quantify viruses in the samples, were calculated for each run on the ABI 7500 (samples were analysed in several batches), and ranged from 0.940030 to 0.996296.

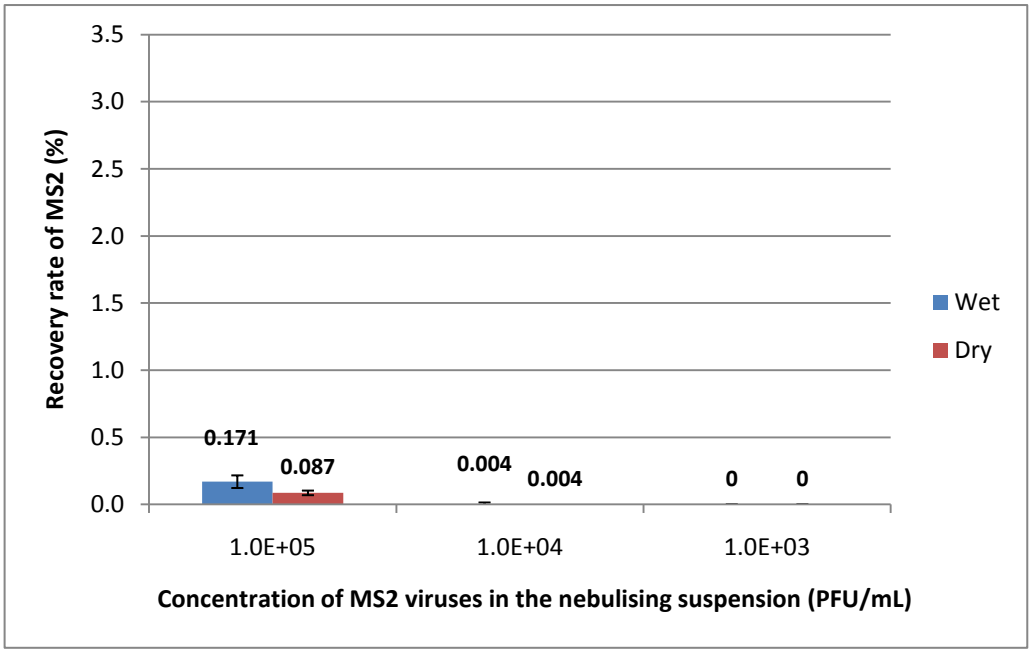


Figure 6-1 Recovery of infectious MS2 from droplets generated from a PBS nebulising suspension of varying MS2 concentrations, as detected by the plaque assay

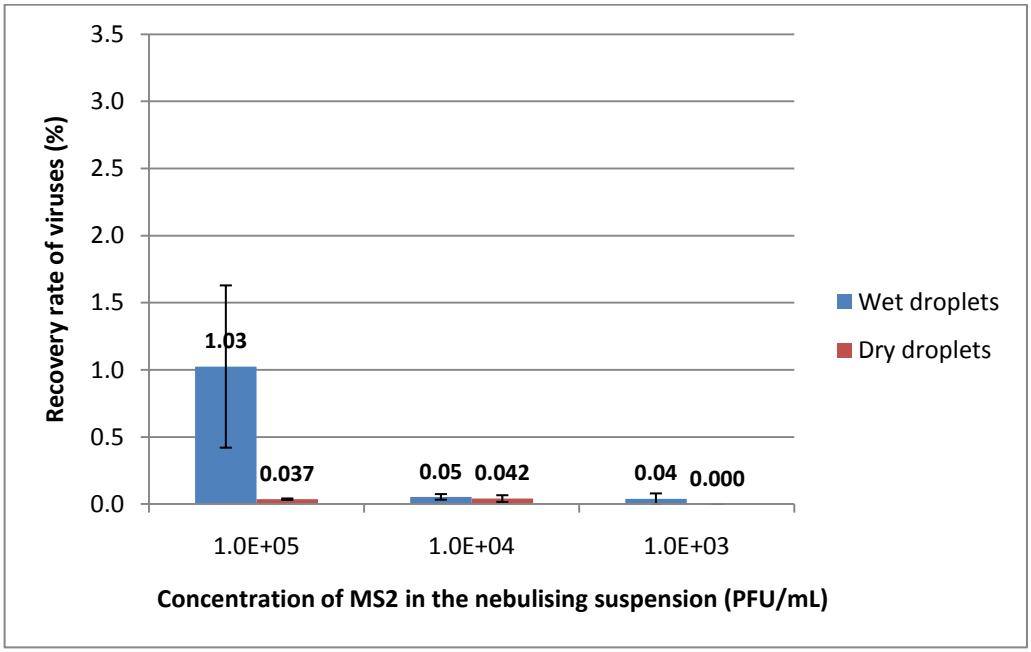


Figure 6-2 Recovery of MS2 RNA copies from aerosols generated from PBS nebulising suspensions of varying MS2 concentrations, as detected by the nested real-time PCR assay

6.4 Results

6.4.1 Effect of varying MS2 concentration in the nebulising suspension on recovery of MS2 from droplets

When the concentration of MS2 was varied in the PBS nebulising suspension, the lower limit of detection for the plaque assay was 10^4 PFU/mL for both wet and dry droplets. When the phage concentration was varied in the artificial mucous suspension, the plaque assay detection limit was a nebulising suspension concentration of 10^4 PFU/mL for the dry droplets and 10^3 PFU/mL for the wet droplets. The limits of the nested real-time PCR assay were similar to that of the plaque assay: when aerosolised from the PBS nebulising suspension of 10^4 PFU/mL, the nested real-time PCR assay could detect MS2 in samples aerosolised in wet and dry droplets. In addition, MS2 was detected from a nebulising suspension of 10^3 PFU/mL from wet droplets. The lower detection limit of the nested real-time PCR assay for MS2 aerosolised from an artificial mucous suspension was a suspension concentration of 10^3 PFU/mL for wet droplets and 10^4 PFU/mL for dry droplets.

Plaque assay results from Chapter 5 that correspond to the PCR results for this Chapter are shown in the following sections to facilitate easier comparison of the two methods.

6.4.1.1 Wet and dry droplets generated from a PBS suspension

The results from the nested real-time PCR assay were similar to the plaque assay results, however, in all circumstances, the nested real-time PCR assay detected more viruses (relative to the original amount aerosolised) than the plaque assay (see figure 6-1 and 6-2).

From a challenge of 10^5 PFU (equivalent to 1.3 x 10^8 RNA copies), an average of 1,324,558 (± 780,684) RNA copies were recovered (1.03 %) from wet droplets. A challenge of 10^4 PFU (1.3 x 10^7 RNA copies) yielded an average recovery of 7013.23 (± 2685) copies, equating to 0.05 %; and an average of 518.13 (± 518.13) copies were recovered from the 10^3 PFU (1.3 x 10^6 RNA copies) challenge (0.04 %).

When dry droplets were aerosolised from the PBS suspension, the average recoveries, as measured by the nested real-time PCR assay, were also higher than the recoveries given by the plaque assay. An average of 48,010 (± 6,859) RNA copies was recovered from the 10^5 PFU challenge (0.037 %). Virus recovery increased slightly to 0.042 % when aerosolised from the 10^4 PFU challenge, with 5408 (± 3193) RNA copies detected by the nested real-time PCR assay. No RNA was detected from the 10^3 PFU challenge.

A two-way ANOVA test showed that no significant effect was observed in the relative recoveries, as measured by the nested real-time PCR assay, by either the concentration of the MS2 in the nebulising suspensions ($p = 0.108$), by the droplet type ($p = 0.112$,) or by the interaction of the two factors ($p = 0.122$).

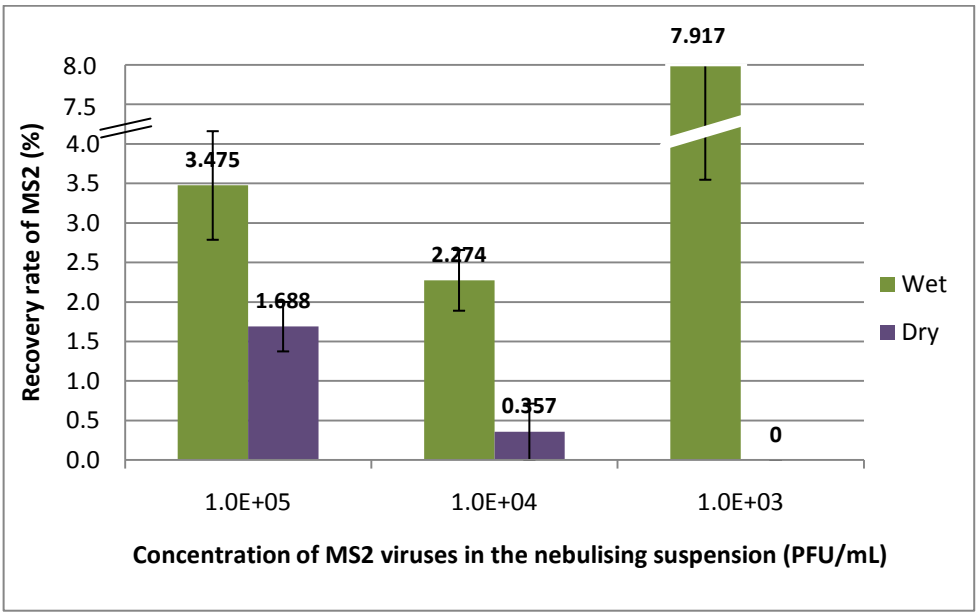


Figure 6-3 Recovery of infectious MS2 from aerosols generated from artificial mucous nebulising suspensions of varying virus concentration, as detected by the plaque assay. This figure was previously shown in Chapter 5 (Figure 5.2)

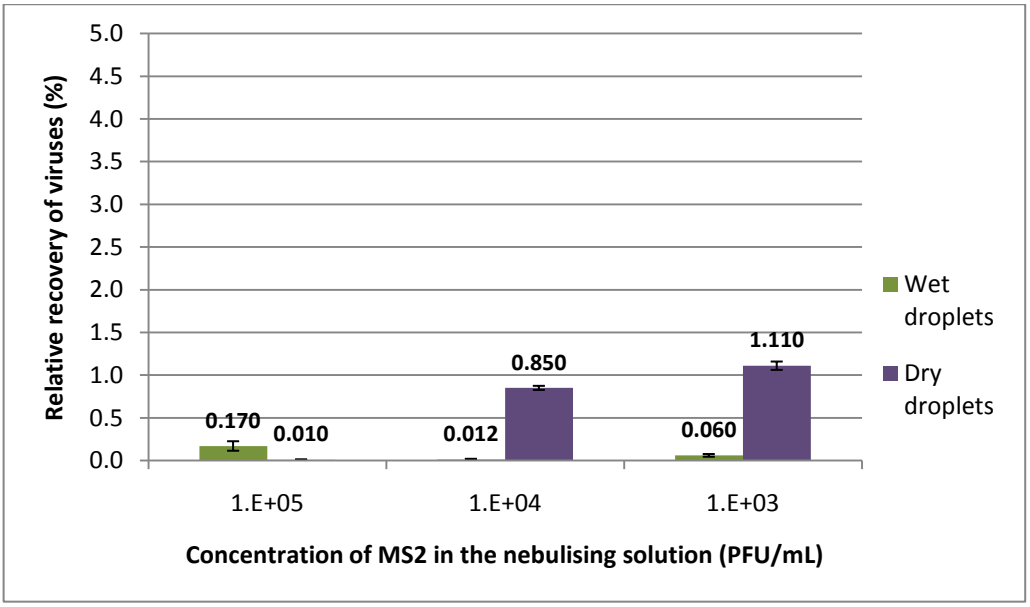


Figure 6-4 Recovery of MS2 RNA copies from droplets generated from an artificial mucous suspension of varying MS2 concentration, as detected by the nested real-time PCR assay

6.4.1.2 Wet and dry droplets generated from an artificial mucous suspension

Similar to the plaque assay results, no explainable trend was apparent when the nested real-time PCR assay was used to detect MS2 in droplets from nebulising suspensions of varying concentrations (see figures 6-3 and 6-4). In the standard experiment for wet droplets, an average of 219,496 (± 71,923) RNA copies, or 0.17 % of the viruses from the original challenge introduced into the chamber was detected. This recovery dropped to 0.012 % from a nebulising suspension of 10^4 PFU/mL, with 1546 (± 1053) RNA copies detected; the recovery level then increased to 0.060%, or 775 (± 204) RNA copies, from the suspension containing 10^3 PFU/mL of MS2.

When aerosolised in dry droplets, the average rate of recovery increased with decreasing virus concentration in the nebulising suspension. This is not to say that the total numbers of RNA copies increased; rather the amount recovered relative to what was introduced into the chamber. The standard experiment yielded very low results (most likely as a result of experimental error) with a recovery rate of 0.0003 %, equivalent to 368 (± 306) RNA copies. The proportion of viruses recovered from the original suspensions of 10^4 PFU/mL increased to 0.068 % with the detection of 8765 (± 3118) RNA copies; this rate increased again for viruses aerosolised from the suspension of 10^3 PFU/mL, with 1222 (± 631) RNA copies detected, a relative recovery of 0.095 %. For the 10^5 PFU/mL challenge, more infectious MS2 was recovered than RNA copies in the dry droplets (in relative terms of recovery). However, for the challenges with lower PFU, relatively more RNA copies were recovered from the original challenge than infectious MS2.

A two-way ANOVA revealed that the concentration of MS2 in the nebulising suspensions in itself did not have a significant effect on the recovery of RNA copies, for either type of droplet ($p = 0.366$). There also was no strong evidence of an effect on recovery by droplet type ($p = 0.342$). However, the interaction of these two factors did have a significant impact on the recoveries of MS2 as detected by the nested real-time PCR assay ($p = 0.009$). This can be seen in the trends which seem opposite to one another: whilst recovery of MS2 increased with decreasing concentration when aerosolised in dry droplets, it decreased when aerosolised in wet droplets.

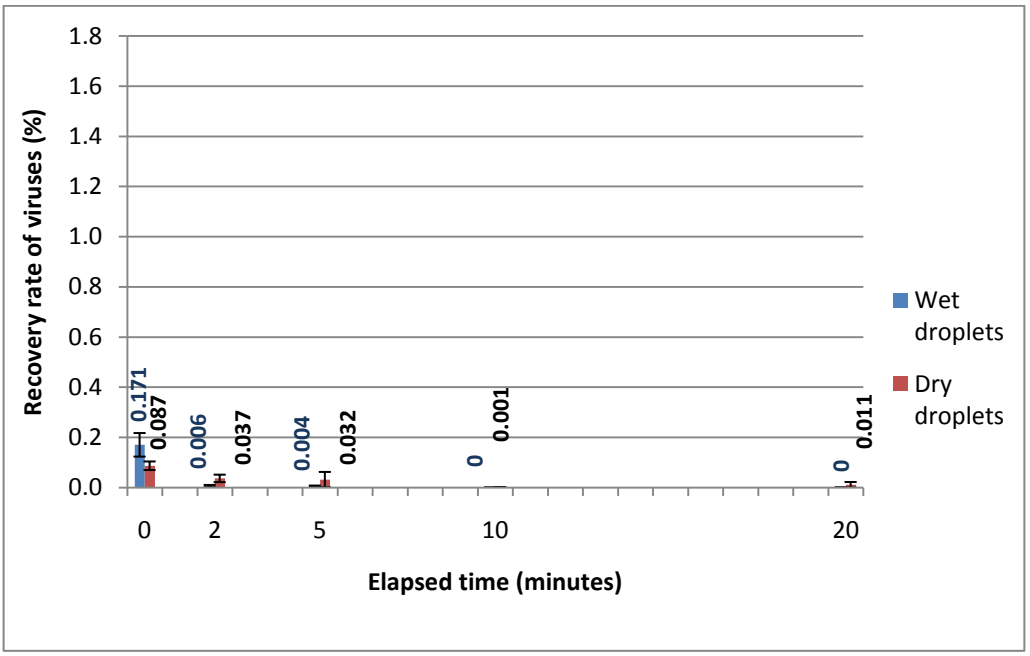


Figure 6-5 Recovery of infectious MS2 from droplets generated from a PBS nebulising suspension and collected after varying elapsed times, detected by the plaque assay

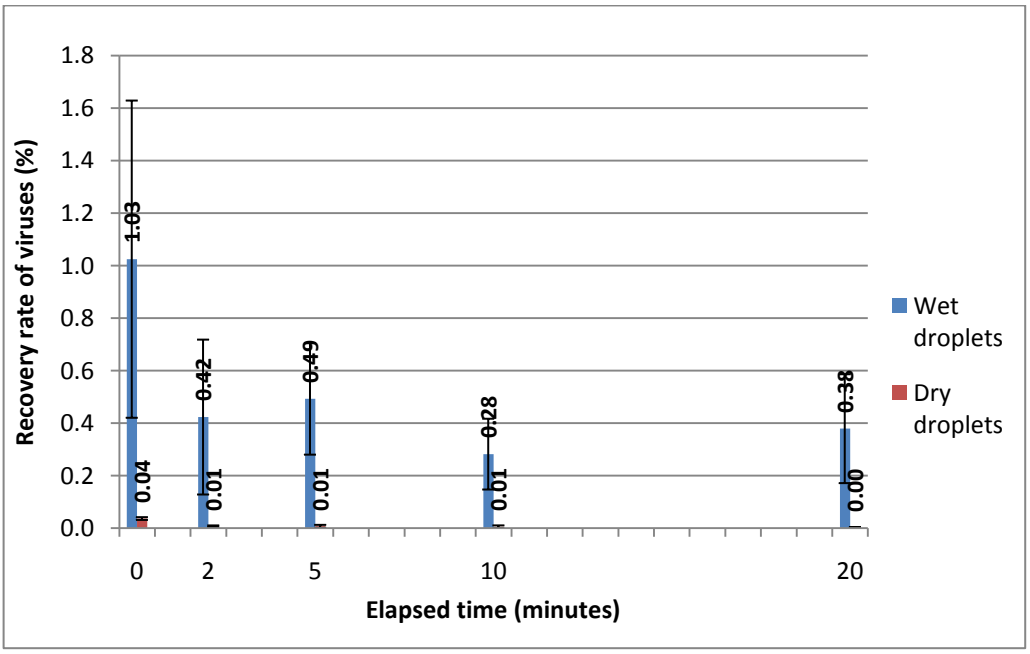


Figure 6-6 Recovery of MS2 RNA copies from droplets generated from a PBS nebulising suspension and collected after varying elapsed times, detected by the nested real-time PCR assay

6.4.2 Effect of varying elapsed time in the chamber on recovery of MS2 from droplets

6.4.2.1 Wet and dry droplets generated from a PBS nebulising suspension

When MS2 was aerosolised in wet droplets, the recovery rates of MS2 RNA copies were higher than those of the infectious particles. When wet droplets were collected immediately after aerosolisation (elapsed time = 0 minutes), 0.171 % of the infectious viruses were recovered from the original challenge by the plaque assay. However, the nested real-time PCR assay detected 1.03 % of the RNA copies, an average of 1,324,558 (± 780,684) RNA copies, from the original challenge. After a time elapse of two minutes, infectious MS2 levels as detected by the plaque assay, dropped to 0.001 %. However, the recovery rate of RNA copies dropped to only 0.42 % when detected by the nested real-time PCR assay, with an average of 547,496 (± 381,113) RNA copies detected. After five minutes elapsed time, the plaque assay showed that levels of infectious viruses dropped to 0.004 %, and then to 0.0002 % after 10 minutes elapsed between aerosolisation and collection. No infectious viruses were detected at the 20 minutes elapsed time point. The nested real-time PCR assay, on the other hand, detected 636,653 (± 274,203) RNA copies after five minutes elapsed, equating to 0.49 % of the original challenge. The virus level then decreased to 0.28 % at ten minutes with 365,052 (± 174,191) RNA copies detected; and at the last time point of 20 minutes elapsed time, the virus recovery rate rose slightly with 267,856 (± 490,294) RNA copies detected (0.38 %).

When aerosolised in dry droplets, the recovery, rate as measured by RNA copies, was comparable to the MS2 infectivity recovery rates. Upon immediate collection, 0.087% of the original challenge remained infectious; whilst 0.04% of the RNA copies were recovered from the original challenge, with 48,010 (± 6,859) RNA copies detected by the nested real-time PCR assay. The level of infectious viruses then dropped after a two minutes elapse to 0.037%; meanwhile, an average of 9,677 (± 4437) RNA copies were detected, this indicated a recovery rate of 0.017% of the RNA copies from the original challenge. Infectivity remained at a similar level after five minutes elapsed: 0.032% of infectious MS2 was recovered as opposed to 0.007% of the RNA copies, which equated to an average of 9,643 (± 7,281) RNA copies. After ten minutes elapsed however, the level of infectious viruses dropped to 0.001%. The nested real-time PCR assay showed that the rate remained at 0.006% of the original RNA copies. Finally, after 20 minutes elapsed, 0.011% of the

original viruses remained infectious, whilst the rate of recovery of the RNA copies was 0.004%, with 4,567 (± 1,751) RNA copies detected.

A two-way ANOVA test showed that the type of droplet did have a significant effect on the relative recovery of the virus, where more RNA copies were detected in the wet droplets ($p = 0.003$). The elapsed time, however, did not have a statistically significant effect on virus detection in either droplet type ($p = 0.518$). Further, the interaction of droplet type and elapsed time together did not affect the virus recovery significantly ($p = 0.605$).

6.4.2.2 Wet and dry droplets generated from an artificial mucous nebulising suspension

The nested real-time PCR assay was able to detect more relative RNA copies than infectious particles when collected after 20 minutes elapsed time (see figure 6-7). Using the plaque assay, 3.474 % of the infectious particles generated from the artificial mucous suspension were recovered initially (data not shown here, see Section 5.3.1); this dropped to zero viruses detected at the 20 minutes time point. However, the nested real-time PCR assay detected 1.70 % of the original RNA copies immediately after release, which then dropped to 0.84 % after a 20 minute elapse time.

When aerosolised in dry droplets, only 0.0003 % of the RNA copies were detected by the PCR. This is probably due to an experimental error. After a 20 minute elapse time, 0.012 % of the RNA copies were recovered. This is in comparison to the plaque assay which measured a relative recovery rate of 1.688 % infectious particles upon immediate collection. No infectious MS2 was detected after 20 minutes had elapsed.

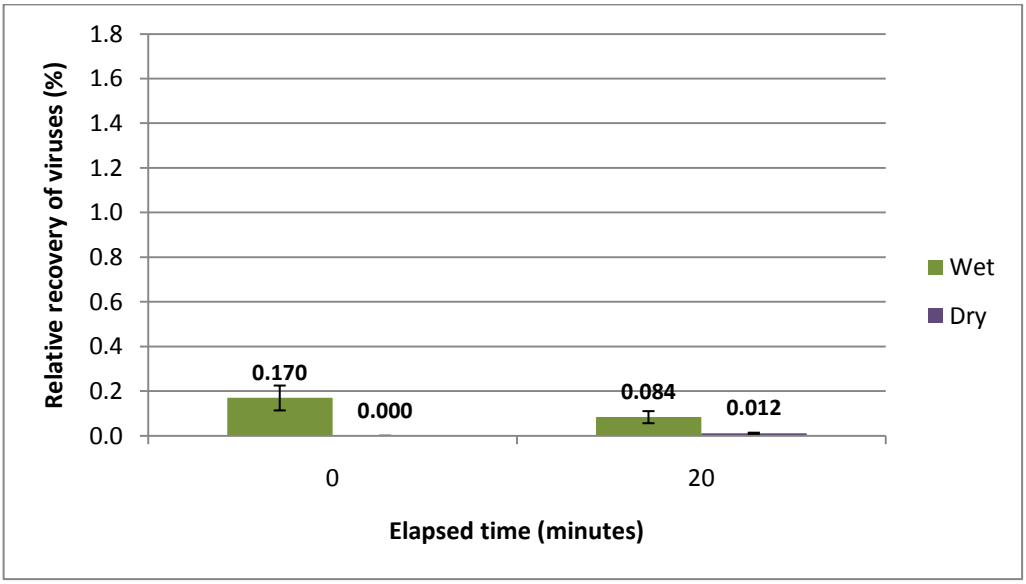


Figure 6-7 Recovery of MS2 RNA copies from droplets generated from an artificial mucous suspension after varying elapsed times, as detected by the nested real-time PCR assay

6.5 Discussion and Conclusions

The nested real-time PCR assay generally favoured the detection of MS2 in droplets. However, considering that there are a lot more viral copies of RNA present in a given sample than viruses which can form a plaque (an estimated 1292 copies to one PFU), this is not surprising. It is of course very likely that we will find more RNA copies than infectious viruses in a sample. Overall, the nested real-time PCR assay results were fairly comparable to the plaque assay in terms of trends against the variables tested. However, when virus concentration in the nebulising suspensions was varied, this was not the case. Whilst the nested real-time PCR assay was able to detect more viruses overall, the patterns of recovery did not fit a predictable trend. The nested real-time PCR assay did exhibit a higher recovery of viruses, with one exception: wet droplets aerosolised from the artificial mucus nebulising suspension had a much higher recovery as measured by infectious plaques. The plaque assay also had a higher recovery of viruses from dry droplets collected from the standard experiment (10^5 PFU/mL) than the recovery as detected by the nested real-time PCR assay; however it is possible that this was an outlier, and due to technical faults. Furthermore, the very high result obtained in the lowest nebulising suspension virus concentration was noteworthy; this sample was tested several times and similar results were obtained. We also showed that MS2 relative recovery from artificial mucous dry droplets increased with decreasing concentration of viruses in the nebulising suspensions. The opposite was observed for wet droplets, where recovery of MS2 decreased with the decreasing concentrations of viruses in the nebulising suspensions. This may indicate that RNA integrity was maintained by the dry droplets more so than the wet droplets, and that this effect was more pronounced with decreasing concentrations.

We showed previously that when held in a PBS nebulising suspension, MS2 bacteriophage could be detected by plaque assay up to ten minutes following aerosolisation in wet droplets, and up to, and possibly beyond, 20 minutes after aerosolisation in dry droplets. The nested real-time PCR assay was able to detect MS2 in the air 20 minutes after aerosolisation, when aerosolised in both wet and dry droplets. Visible trends in the data were evident in this variable, which were similar to the results from the plaque assay. The same drop in virus levels was seen between the zero and the two minute elapsed time points, by both detection assays. The levels of virus remained stable beyond this time point and did not fall much further; this was in contrast to the plaque assay, where the level of infectious viruses decayed at a more rapid rate. In future studies, the elapsed times could be extended to up to hours, to see how long the relative amounts of RNA copies remain

stable, and to see at approximately what endpoint they can still be detected. Again, the comparable levels of viruses aerosolised in wet droplets were much higher than the corresponding experiments where dry droplets were aerosolised.

MS2 infectivity was maintained in droplets generated from the artificial mucous when collected immediately following aerosolisation. However, when MS2 was held for longer than this time, infectivity dropped off completely, as evidenced in Section 6.4.2.2, where the plaque assay was not able to detect any MS2 at elapsed times of ten or twenty minutes. It is possible that there were still infectious viruses present in droplets for up to ten minutes (at two or five minutes), however these time points were not tested. When the nested real-time PCR assay was used to detect MS2 aerosolised from the artificial mucous suspension, however, it was able to detect MS2 up to 20 minutes following aerosolisation for wet droplets, and up to ten minutes for dry droplets. When considering data gathered in Chapter four however, it could be suggested that the effect of the artificial mucous on MS2 infectivity over time, when held on ice, could have contributed to the lack of infectious viruses detected beyond ten minutes. This effect, in combination with decreased viral load in aerosols and the potentially harmful effect of impaction, could have all been responsible for the failure of the plaque assay to detect infectious MS2 in droplets.

Based on our quantification method, we have estimated that there are approximately 1292 RNA copies to each PFU in the stock solution of MS2. It must be noted that we are not attempting to compare these two units directly, as they measure different properties. Whilst we do not have enough information to draw a conclusion as to why there is such a disparity between these two figures, a few assumptions may be inferred. It is possible that the nested real-time PCR assay may be about 1300 times more sensitive than the plaque assay. It is also possible that it takes about 1300 RNA copies to form a plaque. The other possibility is that out of every 1300 (approximate) RNA copies of viruses, only one will be found to be intact and thus able to form a plaque.

The PCR method we have developed here is not a perfect solution to the problems encountered in detecting viruses in droplets; however it is a strong step in the right direction. Clearly, it is not intended that this method be used as a measure of infectivity, as currently, it can give us no information as to the infectivity of the viruses in the samples. As evidenced by the large error and standard deviation values, it is clear that there are still problems with accurately quantifying viruses in air samples. However, we have developed an assay which is very sensitive and can detect viruses in droplets where plaque assays

cannot. The information in this Chapter, as well as the method development, can be applied to the study of respiratory viruses in droplets.

Chapter 7: Detection of influenza viruses in aerosols using the nested real-time PCR assay

7.1 Abstract

The detection of influenza viruses in aerosolised droplets is described in this Chapter. Experiments explored specific variables as determined by the previous results obtained from the MS2 work as discussed in Chapters 4, 5 and 6. Elapsed times and nebulising suspension virus concentrations were varied, but we only investigated their effects on droplets generated from the artificial mucous, using the nested real-time PCR assay to detect RNA copies of the viruses in collected air samples. The data showed that the presence of RNA copies in droplets was higher than expected, and lower detection limits were not reached in the experiments performed here.

7.2 Introduction

Until recently, information regarding the size distribution of aerosols expelled by individuals with a respiratory infection was not available. However, since the appearance of the highly pathogenic avian influenza strain H5N1, an increase in publications has been evident, most likely as a result of increased funding. Some of these publications have described the detection of influenza in simulated droplets using PCR methods (Blachere *et al.*, 2007, Fabian *et al.*, 2009a), and one has successfully detected influenza in droplets created in exhaled breath of infected individuals (Fabian *et al.*, 2008).

Whilst this has certainly contributed to our knowledge, there are still many mechanisms involved in airborne transmission of influenza (and other respiratory diseases) that we do not understand. Without this information, we cannot succeed in the prevention of a worldwide pandemic. Specifically, we must learn more about the extent to which respiratory viruses remain in aerosols, and the physical factors that affect this.

The information gathered in the previous chapters was used to inform a similar study using influenza virus. We showed that when held in both a PBS nebulising suspension and an artificial mucous nebulising solution, MS2 bacteriophage can be detected by the nested

real-time PCR assay up to, and possibly beyond, 20 minutes after aerosolisation from both wet and dry droplets. When MS2 was aerosolised from suspensions of 10^3 PFU/mL, the nested real-time PCR assay could detect MS2 in all four types of droplets (PBS and artificial mucous, wet and dry droplets) except dry droplets aerosolised from the PBS suspension, for which the limit of detection was 10^4 PFU/mL.

Based on this data, it is apparent that artificial mucous does give quite different results in terms of recovery, in comparison to the PBS nebulising suspension. This set of studies examined influenza aerosolised only from an artificial mucous suspension, using the following variables: the effects of droplet type (wet or dry); concentration of virus in the nebulising suspension; and the effect of elapsed time on virus recovery. However, due to the hazards associated with aerosolising influenza viruses, only selected experiments were performed. Furthermore, only the PCR method was used to detect viruses in the collected chamber air. Given the complex nature of cell culture technique required to cultivate influenza virus, this method would have been quite unsuitable for detecting aerosolised virus. The added stresses to the viruses from aerosolisation would most likely have a profound effect on the 'culturability' of the virus, and give poor results. Therefore, only the PCR technique, as developed in Chapter three, was used to detect influenza viruses in aerosols.

The objective of this Chapter was to gain a better understanding of the effect of physical factors on the recovery of influenza virus in aerosols, applying information gathered in chapters four, five and six to similar studies.

7.3 Methods

Aerosol delivery and collection and sample processing were performed as described in Chapter two, Sections 2.3 and 2.4. As informed by the results reported in Chapters four and five, only artificial mucous was used as a nebulising suspension in this study, to more accurately simulate a real-life situation, where the mucous of infected individuals is more viscous than a water-like suspension (like PBS). The single stage BioStage® sampler was used, as the samples were analysed only by the nested real-time PCR assay. Influenza virus stocks were provided by the virology laboratory. These viruses were propagated in canine kidney cells (MDCK), and titrated using both plaque assay and $TCID_{50}$ assay.

Samples were collected and extracted immediately after the conclusion of each experiment to minimise any loss of viral RNA. For the experiments where influenza recovery was expected to be low, entire samples were used for RNA extraction in order to maximise the chance of detecting the virus.

Quantification of the samples by the nested real-time PCR assay is described in Chapter three with some modifications. The first step of the nested PCR was performed using the Eppendorf Gradient S Mastercycler (Eppendorf, Hamburg, Germany) with the following cycling conditions: a 50 °C hold for 30 minutes; a 94 °C hold for 2 minutes; 20 cycles of 94 °C for 15 seconds, 55 °C for 30 seconds and 68 °C for 60 seconds, and a further extension step of 68 °C for 5 minutes. The second step of the nested real-time PCR assay (real-time PCR) was performed with Applied Biosystems fast PCR mastermix, which did not include a reverse transcriptase step, and used the following cycling conditions: 95 °C for 20 seconds; 40 cycles of 95 °C for 3 seconds and 60 °C for 30 seconds. Data (fluorescent signal) was collected during the 60 °C step of each cycle.

All aerosol challenges were introduced for 30 seconds, at flow rates of six L/min. Both types of aerosol (wet and dry) were used to examine virus detection. The influenza virus used was an H3N2 strain (A/Brisbane/10/2007).

The variables explored in this part of the study were: elapsed times of 0 minutes, 10 minutes and 20 minutes; and nebulising suspension viral concentrations of 10^5, 10^4, 10^3 and 10^2 PFU/mL (wet droplets only). These concentrations were chosen to align with the concentrations of the MS2 in the previous chapters; however the 50% tissue culture infective dose per mL ($TCID_{50}$/mL) measurements were also recorded in order to allow analysis of our results in relation to the literature (where influenza virus titres are often cited in this format). $TCID_{50}$ is a measure of the dilution of viruses where 50% of cell cultures exhibit a cytopathic effect (CPE); this is a commonly used method of quantification in clinical studies. The stock concentration of the influenza strain used in these experiments was calculated as: 9.3×10^5 PFU/mL and $10^{6.22}$ $TCID_{50}$/mL.

Samples collected from the experiments were screened during the progression of the study and quantification was performed at the conclusion of the work. For quantitative analysis, the standard curves used were performed with each run from the first step of the assay so as to obtain the best results possible. The correlation coefficients of the standard curves for all assays (as samples were analysed in groups) were shown to range from 0.991512 to 0.998226.

7.4 Results

7.4.1 Effect of varying influenza concentration in the nebulising suspension on recovery of influenza, from wet and dry droplets

Influenza virus was detected in aerosols following aerosolisation from the nebulising suspensions, at all concentrations tested. The relative rate of detection from nebulising suspensions of differing concentrations was quite stable for both wet droplets and dry droplets (see figure 7-1). However, there was no clear trend present.

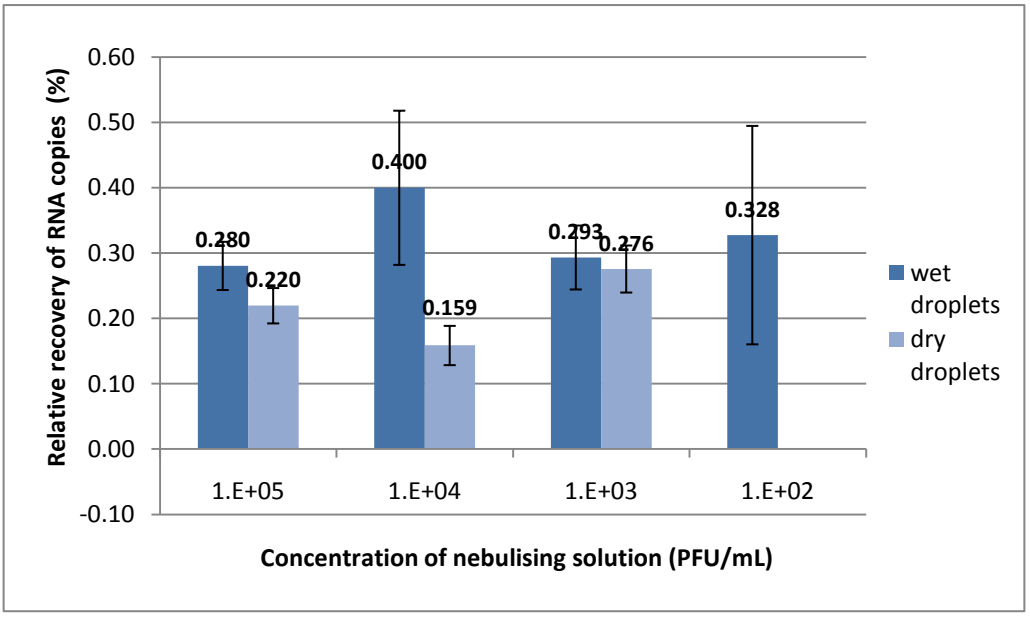


Figure 7-1 Relative recovery of influenza viruses, for both wet and dry droplets, from nebulising suspensions of varying concentrations

When aerosolised in wet droplets, the standard experiment, using a nebulising suspension with a concentration of 10^5 PFU/mL influenza viruses (estimated to be equivalent to 1,224,616,240 RNA copies) yielded a 0.280 % recovery from the original challenge, when collected immediately following aerosolisation; this was an average of 3,434,479 (± 451,524) RNA copies. From a nebulising suspension with a concentration of 10^4 PFU/mL, 911,296 (± 144,481) RNA copies were detected, a recovery rate of 0.744 %. This was very high and it is most likely that this was due to a technical error. When aerosolised from a nebulising suspension with a concentration of 10^3 PFU/mL influenza viruses, an average of 35,920 (± 5,991) RNA copies were detected, which was 0.293 % of

the original challenge. The relative recovery rate increased to 0.328 % with an average of 4,012 (± 2047) RNA copies detected from a nebulising suspension of 10^2 PFU/mL.

The recovery of influenza virus from the dry droplets also lacked an obvious trend. From the standard nebulising suspension of 10^5 PFU/mL, an average of 2,688,117 (± 331,951) RNA copies was recovered, which was equivalent to a 0.22 % recovery. This rate decreased to 0.159 % from the suspension with a concentration of 10^4 PFU/mL, equating to 194,309 (± 36,787) RNA copies, and then increased to 0.276 % from the nebulising suspension of the lowest concentration tested, with an average of 33,761 (± 4,408) RNA copies. A lower dilution was not tested for dry droplets as recovery was not expected, based on data observed from the previous MS2 studies.

A two-way ANOVA test showed that the concentration of viruses in the nebulising solution did not have a significant influence on the relative recovery of the viruses ($p = 0.86$). However, the droplet type did have a significant effect on the recovery of viruses ($p = 0.006$); specifically, viruses aerosolised in dry droplets were present in fewer numbers. The interaction of the two factors (virus concentration and droplet type) also had a statistically significant impact ($p = 0.007$).

7.4.2 Effect of varying elapsed time in the chamber on recovery of influenza, from wet and dry droplets

Influenza virus was detected in aerosols up to 20 minutes after aerosolisation, for both wet and dry droplets. In contrast to our previous results using MS2, the recovery of detectable viruses over elapsed time was somewhat favoured by aerosolisation in dry droplets, as opposed to wet droplets. Immediately after the aerosolisation of the nebulising suspension containing influenza, the number of RNA copies recovered was 3,434,479 (± 451,524) from the wet droplets and 2,688,117 (± 331,951) from dry droplets. These viruses were recovered from an original challenge of 1,224,616,240 calculated copies, so the relative recoveries were 0.280 % for wet droplets and 0.220 % for dry droplets (see figure 7-2).

Virus numbers collected ten minutes after aerosolisation remained quite high. For wet droplets, the average number of RNA copies detected was 3,224,659 (± 2,538,394) which was a recovery rate of 0.263 %, whereas for the dry droplets the average number of RNA copies recovered after ten minutes was 2,735,845 (± 97,890), which was a small relative increase from 0.220 % to 0.223 %.

After 20 minutes had elapsed between aerosolisation and collection, there was still a relatively large number of viruses suspended in the air from the wet droplets, with 1,426,660 (± 797,284) RNA copies, a relative recovery of 0.116 %. When aerosolised from the dry droplets, the virus numbers did not decrease as much as for the wet droplets over time, and an average of 1,964,585 (± 821,269) RNA copies was detected, which was a recovery of 0.160 % from the original challenge.

A two-way ANOVA test on this data showed that neither the droplet type nor the elapsed time significantly affected the recovery of viruses in droplets ($p = 0.810$ and 0.443 respectively).

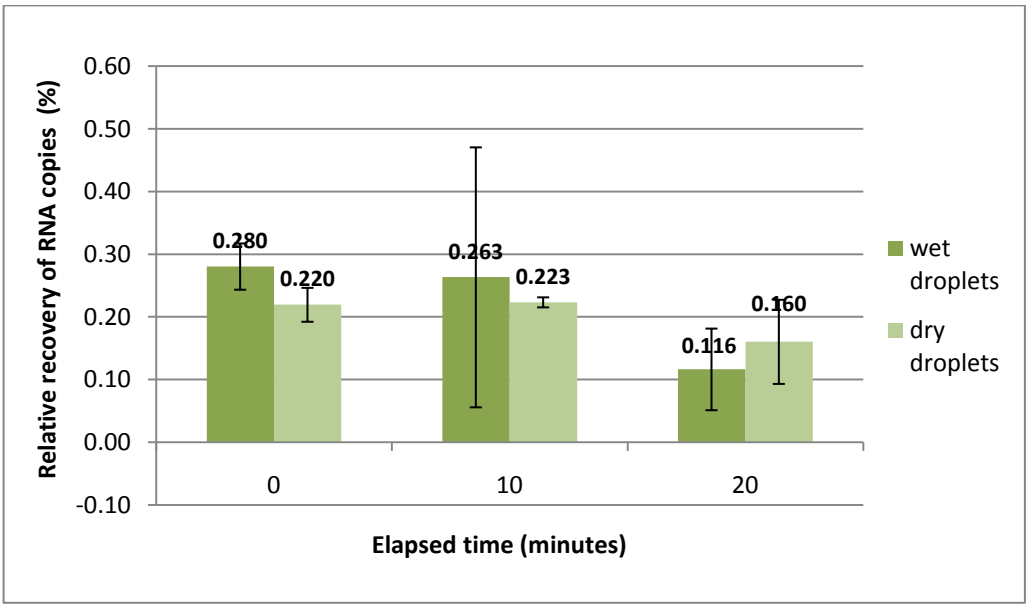


Figure 7-2 Influenza viruses detected from aerosols, collected after different elapsed times

7.5 Discussion and Conclusions

The results obtained in this study initially were quite surprising in terms of the overall number of RNA copies detected; however, quantification of the influenza virus stock used in the aerosol experiments revealed that there were far more copies of the viral RNA (ie viruses) in the original stocks than expected. It is widely held that cell culture techniques underestimate the amount of infectious viruses present; that the count achieved from this method grossly underestimates the true amount of infectious viruses in a given sample is more than likely. Therefore, a nebulising suspension with a measured concentration of

10^5 PFU/mL will most likely contain many more infectious viruses than that expected from this quantification method. It must also be considered that publications and reports utilising these cell culture methods to describe influenza and its pathology were most likely underestimations also, for the same reasons. Thus, it would follow that the concentrations/numbers of viruses in the respiratory secretions of infected individuals may be much higher than was originally thought.

Overall, the proportions of influenza viruses that were detected by the PCR method were comparable to the MS2 results. The recovery rates of influenza viruses from aerosols, based on the estimated RNA copy numbers, ranged from 0.116 % to 0.328 % (excluding the outlier which was 0.744 %). Consistent with the results from the previous chapters, the wet droplets generally had a higher recovery rate in the experiments, than the dry droplets.

Whilst the similarities in recovery rates for MS2 and influenza may seem surprising given that MS2 is a non-enveloped virus and influenza is an enveloped virus, this can be explained by the relative humidity levels during the experiments. Generally, viruses with lipid envelopes tend to have higher inactivation rates at higher RH (above 40 %), whilst some viruses which lack a lipid envelope have been shown to have higher inactivation rates at lower RH (Tellier, 2006). Tseng and colleagues (2005) showed, however, that when collected in a single-stage Andersen sampler (the same that was used in this study), MS2 was not significantly affected by RH, and maintained its recovery rate at RH of 20 %, 55 % and 85 %. Furthermore, RH was held at 45-55 % during this study, which could be considered to be on the overlap at which enveloped and non-enveloped viruses have similar recovery rates. If lower and higher RH were tested here, widely varying recovery rates would have probably been observed.

The concentrations of the nebulising suspensions were calculated based on quantification of the influenza stock used, which was previously established by cell culture methods. The results are expressed here as relative recovery of RNA copies from that original stock, as the quantification difference between the plaque forming units and the RNA copies was rather substantial: a stock of 100,000 PFU/mL was equal to 1,224,616,240 RNA copies; this is equivalent to approximately 12,246 RNA copies per plaque forming unit. Considering this figure, the recovery rates encountered here (0.116 % to 0.328 %), although appearing to be quite small, actually equate to a high number of viruses, in the order of 10^6 RNA copies in some experiments.

Originally, nebulising suspensions with concentrations ranging from 10^5 PFU/mL to 10^3 PFU/mL were tested, however after discovering relatively high levels of virus in aerosols at the lowest concentration (10^3 PFU/mL) a further dilution to 10^2 PFU/mL was tested, revealing that viruses can still be detected from a seemingly low dilution. Based on the data observed in the MS2 studies, it was expected that no viruses would be detected at this concentration from dry droplets, as the numbers of viruses from the dry droplets were consistently lower than those of the wet droplets. Considering this data however, it is possible that there would have in fact been detectable viruses in dry droplets when aerosolised from a nebulising suspension of 10^2 PFU/mL. It is also feasible that aerosols from nebulising suspensions of even lower virus concentrations would also have contained detectable numbers of viruses. Alternatively, influenza viruses may be much 'tougher' than MS2, that is, their RNA may not break down at a similar rate.

The peak concentrations of influenza virus in nasopharyngeal samples at 48 hours post infection with seasonal human influenza has been found to be 10^3 to 10^7 $TCID_{50}$/mL (Murphy *et al.*, 1973). The experiments conducted in this research generated droplets from nebulising suspensions that were within this virus concentration range, so we can infer that the results are representative of a real-case scenario. This, however, does not take into account all the influencing factors, such as the reception of the aerosols by a susceptible host and subsequent infection. Further, if considering an outdoor environment, there would be many other factors involved such as dispersal, Brownian motion, and so on.

The amount of influenza RNA copies detected after elapsed times of 10 and 20 minutes were relatively high and more stable than was expected; this is especially the case for the dry droplets, where, after 10 minutes, the level of detectable virus did not decrease. At 20 minutes, the level of detectable viruses had only dropped 27 % below the level of viruses collected immediately after aerosolisation, compared to a 58 % observed in viruses for wet droplets. This would suggest that dry droplets somehow preserve the amount of detectable viruses, although the initial amount of viruses detected after aerosolisation is lower in dry droplets than wet droplets. Perhaps these results should not have been surprising: data from previous studies, dating back decades, shows that influenza in aerosols can remain viable up to 24 hours after aerosolisation at low levels of relative humidity (<55 %) (Loosli, 1943, Hemmes *et al.*, 1960).

Further exploration of the variables used here would be beneficial to defining the limits of influenza detection in droplets. In particular, further investigation into the time that the

virus could survive would be interesting. This may include extension of elapsed times up to several hours after aerosolisation.

In conclusion, the detection of influenza viruses by the nested real-time PCR assay was successful and higher than anticipated. Moreover, the persistence of influenza viruses with varying environmental conditions tested here was note-worthy.

Chapter 8: Summary and Conclusions

8.1 Revisiting the hypothesis and aims

This research was undertaken in order to interrogate the hypothesis: "That RNA viruses may be quantitatively detected in aerosols using a purpose-designed assay system that is sufficiently sensitive". It was further hypothesised that the recovery of virus particles, modelled initially using MS2 bacteriophage and subsequently tested for influenza virus, would vary with certain physical factors, such as the temperature, the nature (viscosity) of the suspending matrix, the moisture content of the aerosol droplets, and the time interval between aerosolisation and collection of the droplets.

The hypothesis was tested by addressing the following three major aims.

The first aim was to develop a sensitive nested real-time PCR assay to detect and quantify potentially stressed or damaged viruses in droplets. Nested real-time PCR assays were successfully developed for bacteriophage MS2 as well as for four respiratory viruses: influenza A (H1N1 and H3N2 subtypes); influenza B; parainfluenza virus (subtype 1); and human respiratory syncytial virus.

The second aim was to examine the behaviour and recovery of aerosolised MS2 within a sealed chamber, including individual droplet size, limiting physical factors (i.e. times held in the chamber and concentrations of viruses in the suspensions), and the relative recovery of viruses in various conditions. The PCR technique developed in the first objective was applied to samples, collected specifically for this purpose, and results were reviewed in parallel with the traditional culture techniques. Both liquid and mucous-like suspensions were compared to determine whether they were significantly different, and to permit a closer simulation of real-life situations. Bacteriophage MS2 was selected as the surrogate for respiratory viruses due to its similar size and shape, and prior use in similar studies. MS2 was expected to behave in a similar manner to the respiratory viruses.

The final aim was to apply knowledge gained from the bacteriophage experiments, to similar experiments with influenza. The sensitive nested real-time PCR assay for influenza, developed for aim one, was used to detect the respiratory viruses and their recovery patterns

.

8.1.1 Hypothesis

The hypothesis was proven: RNA viruses were detected and quantified in aerosols using a successfully designed nested PCR technique, described in Chapter three of this document and also in Perrott *et al*., 2009. The secondary hypothesis was also proven, in that, using this sensitive technique, virus recovery was able to be tested, using MS2 and then respiratory viruses, and was shown to vary with certain physical characteristics.

8.2 Summary of findings

We characterised four different droplet types and found that each droplet type had a significantly different effect on the persistence of viruses in the droplets. Most importantly, we demonstrated that the artificial mucous had a protective effect on virus infectivity, under certain conditions. We also showed that MS2 could be recovered from droplets, with relative recovery rates of up to 3.474 % as detected by the plaque assay, and up to 1.110 % as detected by the PCR assay, whilst influenza virus was detected at recovery rates of up to 0.400 %, as detected by the nested real-time PCR assay.

Sensitive, quantitative, nested, real-time PCR assays were developed as part of this study, and successfully applied to aerosol studies using MS2 bacteriophage and influenza virus. The assays were used in combination with synthetic RNA controls for preparation of standard curves against which the unknown samples were quantified.

We showed that when held in a PBS nebulising suspension, infectious MS2 bacteriophage could be detected by the plaque assay up to ten minutes following aerosolisation in wet droplets, and up to, and possibly beyond, 20 minutes after aerosolisation in dry droplets. When MS2 was aerosolised from the artificial mucus suspension, it was not detected in the air by the plaque assay at ten minutes or 20 minutes following aerosolisation (from wet or dry droplets). However, we believe this is due to the result of a combination of effects including: the decay over time of MS2 virus infectivity when held in artificial mucous suspension on ice; the damage inflicted by impaction onto the agar; and the reduced viral load in the droplets.

When the concentration of MS2 was varied in the PBS nebulising suspension, the lower limit of detection of infectious viruses, using the plaque assay, was 10^4 PFU/mL for both wet and dry droplets. When the concentration was varied in the artificial mucus

suspension, the plaque assay detection limit was a nebulising suspension virus concentration of 10^4 PFU/mL for the dry droplets and 10^3 PFU/mL for the wet droplets.

The influenza virus study provided surprisingly robust and stable results, considering prior assumptions that it is a labile virus. The recovery of influenza was neither higher nor lower than that of the MS2 bacteriophage, overall. Rather, it was much more consistent with respect to the levels of virus remaining in the droplets. Whether or not this is due to the nature of virus stability is a question that cannot be answered absolutely by this study. It is possible that the differences in the efficacies of the nested real-time PCR assays may have contributed to this finding. Indeed, it has been shown that overall, the influenza nested real-time PCR assay was more robust than the MS2 nested real-time PCR assay, which although sensitive, would often not follow the log patterns one would expect to observe in real-time PCR when a 10-fold series dilution is amplified (where 1 log is equal to approximately 3.3 cycles). This is evidenced in Chapter three: contrastingly, the influenza assay always followed this pattern flawlessly. Moreover, when the standard curves were used to quantify the samples, the correlation coefficients of the MS2 assay standards were lower than those of the influenza standards, although both were satisfactorily high: for the MS2 nested real-time PCR assays the correlation coefficients ranged from 0.940030 to 0.996296; for the influenza nested real-time PCR assays, the correlation coefficients ranged from 0.991512 to 0.998226.

8.3 Significance of findings

The differences between the PBS nebulising suspension and the artificial mucous suspension were interesting: it was clear that the artificial mucous suspension had a protective effect on the MS2 infectivity on immediate release. Based on the standard experiment, more than 20 times the amount of viruses remained infectious (intact) when aerosolised from the artificial mucous suspension in comparison to droplets produced from the PBS suspension.

It may follow that the physical properties of human mucous will have a protective effect on the viruses. However, when examining the results from the assay assessing infectivity in different suspensions at different time points and temperatures, it would seem that MS2 viruses do not remain infectious for long when held in artificial mucous on ice. During all aerosolisation experiments, the nebulising suspensions containing the viruses were kept on

ice between replicates, as it was assumed that this would preserve the virus levels during the waiting periods. However, it seems that the opposite was true. The results from the static experiment described in Chapter four support this theory. Section 4.3.4: data showed that the level of MS2 infectivity dropped dramatically over time, when held in an artificial mucous suspension on ice. This experiment was undertaken after the MS2 aerosolisation experiments had been completed, so we were not aware of this effect until after data had been collected.

This study has shown that influenza can be detected in very small droplets, in rather large numbers. However, it must be noted that the airborne route of infection will be much more limited than is suggested by these results. Morawska stated that aside from external and environmental factors that influence the fate of a particle, the fate of *viruses* within an airborne particle will depend on: the source and mechanism of their expulsion; their ability to 'survive' the physical factors that they face during aerosolisation; and by inherent characteristics of the virus in question (e.g. whether or not is has an envelope) (Morawska, 2006).

This study sought to address the issue of to what extent viruses could survive given optimal conditions. It did not take into account all of the numerous factors which might influence aerial transmission including, but not limited to: bioaerosol production (location of virus in the respiratory tract; production of suitable droplets which viruses can be carried within, etc); transport (environmental factors such as temperature, relative humidity, UV desiccation etc); and reception of aerosols (deposition of droplets, location of deposition, ability of viruses to bind to, infect and replicate in cells). In other words, aside from the factors we have investigated here, the virus needs to also overcome the aerosolisation process, the transport process, remain infectious and find a suitable host in which to initiate disease.

The size of a particle is dictated by the process that leads to its generation, and thus is dependent on its source. In addition, research has shown that the immunological effect on a host when a bioaerosol is inhaled is dependent on the size of that particle, and that submicrometre and ultrafine particles have a much more significant effect (Hogan *et al.*, 2005). Hence, the size of the droplet is the most important factor in droplet fate and thus in airborne infection (Morawska, 2006).

In this study, we examined small droplets with a range of diameters spanning 0.65 - 2.1 µm, which is consistent with the range of respiratory droplets as reported by several studies

(Knight, 1973, Erdal and Esmen, 1995, Kowalski and Bahnfleth, 1998, Morawska *et al.*, 2008). It is the transmission and recovery of intact viruses within these droplets, which become droplet nuclei, that pertains to the aerosol spread of respiratory viruses. We have shown that these droplets can potentially be vehicles of virus transmission, notwithstanding the other factors which affect airborne infection (as mentioned above). Furthermore, droplets that equilibrate to a size smaller in diameter than 0.65 µm were not considered here, as they were not within the size range of the Andersen sampler. This does not mean that the viruses are not intact, but that our limit of detection cannot provide for such a small particle.

Interestingly, the UV-APS showed that for each of the four types of droplets, there was a significant proportion of droplets which also fell outside of the detection range (0.5 µm in diameter). Whether or not these submicrometre droplets can harbour infectious viruses cannot be answered here, but certainly is a future research direction that must be considered.

The differences seen between the MS2 and influenza relative RNA copies are quite substantial: for influenza, a stock of 100,000 PFU/mL was equal to 1,224,616,240 RNA copies; this is equivalent to approximately 12,246 RNA copies per plaque forming unit. However for MS2, the ratio of plaque forming units to RNA copies was 1:1292. This is a 10-fold difference, and more importantly, highlights the inefficiencies of the plaque assay for quantification of influenza virus. Furthermore, when we consider that the virus titres in the nasopharyngeal washes of infected individuals, normally quantified using the tissue culture infective dose method, can be up to $10^{4.8}$ $TCID_{50}$/mL (Murphy *et al.*, 1973), this is conclusive evidence that these infected individuals potentially have the capacity to shed virus, in very high numbers. Whilst we cannot quantify this accurately here, due to the fact that tissue culture dose methods and an estimation of RNA copies do not correspond to one another because of the fundamental difference in their assay principles, we can infer that the numbers of infectious (intact) viruses in previous studies may be much higher than previously thought.

Influenza infects columnar epithelial cells of the respiratory tract, and can infect both the upper respiratory tract and the lower respiratory tract (Bridges *et al.*, 2003). Studies employing histological techniques showed that influenza (in general) infects the bronchial tree, and that ciliated cells are preferred targets to goblet cells in the tracheal epithelium (Nicholls *et al.*, 2008). This information, in combination with the above data as well as our findings, can be used to give a better understanding of the likelihood of production of virus-

laden droplets from an infected host. This information can also help to predict the outcome of bioaerosol reception by a suitable host, in terms of the location of deposition of a virus-laden aerosol (depending mostly on size of the droplet) and the potential site of infection.

Interestingly, H5N1 virus is largely confined to the tracheal epithelial cells and alveoli in the lungs of infected individuals (Korteweg and Gu, 2008). Shinya and colleagues showed that avian influenza viruses (a H5N1 strain from Vietnam, a H4N6 strain from Czechoslovakia and a H3N2 strain from Mongolia) bound extensively to alveolar cells and less so to bronchial epithelial cells; this is in contrast to human isolates of influenza A (H1N1 and H3N2, both isolated in Japan) which show preferential binding to bronchial epithelial cells over alveolar cells. This is one possible explanation for the difference in the transmission of the two types of influenza, where avian influenza does not spread readily between humans. Evidence has shown that highly pathogenic avian influenza virus (HPAIV) has tropism for the lower respiratory tract. The lack of efficient transmission of HPAIV may not only suggest that reception of the bioaerosol is to blame, given that it favours binding to the epithelial cells in the lower respiratory tract, but also suggests that the bioaerosols created from the lower respiratory tract may not be an ideal vehicle for influenza transmission, in comparison with droplets that are generated from the upper respiratory tract. However this data is not yet conclusive so we cannot rule out other explanations.

Some problems were experienced in the course of this study. In particular, the logistics of carrying out such experiments, where there were so many variable factors, that it was quite difficult to obtain results which were primarily affected by changes in those variables. This was done to the best of our abilities; however some problems remained. For example, some studies have shown that aerosolisation in the Collison can affect the infectivity of some organisms. This has been disproven for some organisms, but not for influenza. To overcome this, we tried two methods: firstly, we kept the nebulising suspension on ice for the duration of experiments when it was not being used; secondly, only a few millilitres of the suspension was used at a time, and was topped up with the “fresh” suspension (also kept on ice) after each replicate was performed. We did not consider it reasonable to use an entirely new suspension for each replicate, as the preparation of a new suspension could mean a different resulting concentration (if only by pipetting error). As a result, decay of the infective viruses collected after aerosolisation was evidenced in all experiments for each subsequent replicate, for both MS2 bacteriophage and influenza virus.

We were also limited in the number of experiments that could be performed, due to the sheer volume of sample processing for each experiment. It was also for this reason that we were unable to perform more than three replicates for each experiment.

In addition to contributing to knowledge in the field of influenza transmission, this study has developed several methods for studying both bacteriophage and respiratory viruses in air samples. Methods developed include: sensitive, quantitative nested real-time PCR assays for influenza A (H3N2 and H1N1), influenza B, parainfluenza and RSV as well as MS2 bacteriophage; aerosol collection methods for collecting bacteriophage using host-overlay agar plates; and an aerosol collection method for pathogenic viruses, suitable for applying PCR analysis.

This study is the first to our knowledge to have extensively investigated factors that influence virus integrity and detection. Although other papers claim to have done this, the outputs of such studies have not fully addressed the knowledge gaps. This study aimed to elucidate some of these gaps in order to assist further work in this area. It is by no means a body of work that completely answers all remaining questions; however, we believe the knowledge gathered here is an important step towards the gaining of a better understanding of the airborne transmission of influenza, and thus the prevention of a threatening influenza pandemic.

8.4 Future directions

The research here can be elaborated upon to further investigate the matter and provide answers to much needed questions. Specifically, the study of longer elapsed times can be explored, to determine how likely influenza viruses are able to be recovered after longer times, possibly up to hours after aerosolisation. Furthermore, lower concentrations of influenza viruses should be tested to determine the minimum virus concentration in a nebulising suspension that is required to detect viruses from the resulting droplets.

Another area of this research which can be greatly developed is the application of the nested real-time PCR assay, and its ability to quantify the number of virus particles in a sample. To reduce inconsistencies and increase the reliability and accuracies of the assays, it would be valuable to further improve them; for example, converting each assay into a single step assay may reduce the likelihood of introducing contamination in the samples.

Consequently, the improvement of the assays may enable closer correlation of the results with the plaque assay results.

There is much more work that can be undertaken in this field of research. This study has filled some gaps in terms of developing the methods needed to detect influenza virus successfully from aerosols, and in terms of determining detection limits. Additionally, we have identified physical factors which limit the persistence of influenza virus in droplets. The results gathered here can be applied to research the persistence of other respiratory diseases, including parainfluenza viruses and respiratory syncytial viruses, as well as further investigation of influenza viruses in aerosols.

Chapter 9: References

Adams, M. H. 1959. *Bacteriophages,* New York, Interscience Publishers, Inc.

Agranovski, I. E. 2007. Personal Sampler for Viable Airborne Microorganisms: Main Development Stages. *CLEAN – Soil, Air, Water,* 35, 111-117.

Agranovski, I. E., Safatov, A. S., Borodulin, A. I., Pyankov, O. V., Petrishchenko, V. A., Sergeev, A. N., Sergeev, A. A., Agranovski, V. & Grinshpun, S. A. 2005. New personal sampler for viable airborne viruses: feasibility study. *J Aerosol Sci,* 36, 609-617.

Agranovski, I. E., Safatov, A. S., Sergeev, A. A., Pyankov, O. V., Petrishchenko, V. A., Mikheev, M. V. & Sergeev, A. N. 2006. Rapid detection of airborne viruses by personal bioaerosol sampler combined with the PCR device. *Atmos Environ,* 40, 3924-3929.

Amann, R. I., Ludwig, W. & Schleifer, K. H. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev,* 59, 143-169.

Anonymous 1995. Update: Influenza activity--Worldwide, 1995. *MMWR. Morbidity and Mortality Weekly Report,* 44, 644.

Atmar, R. L., Baxter, B. D., Dominguez, E. A. & Taber, L. H. 1996. Comparison of reverse transcription-PCR with tissue culture and other rapid diagnostic assays for detection of type A influenza virus. *J Clin Microbiol,* 34, 2604-2606.

Australian Government Department of Health and Ageing. 2010. *Australian Influenza Surveillance Report* [Online]. Available: http://www.health.gov.au/internet/main/publishing.nsf/content/cda-surveil-ozflu-flucurr.htm [Accessed November 21 2010].

Bae, H.-G., Nitsche, A., Teichmann, A., Biel, S. S. & Niedrig, M. 2003. Detection of yellow fever virus: a comparison of quantitative real-time PCR and plaque assay. *J Virol Methods,* 110, 185-191.

Barker, J. & Jones, M. V. 2005. The potential spread of infection caused by aerosol contamination of surfaces after flushing a domestic toilet. *J Appl Microbiol,* 99, 339-347.

Benbough, J. E. 1971. Some factors affecting the survival of airborne viruses. *J Gen Virol,* 10, 209-220.

Blachere, F. M., Lindsley, W. G., Slaven, J. E., Green, B. J., Anderson, S. E., Chen, B. T. & Beezhold, D. H. 2007. Bioaerosol sampling for the detection of aerosolized influenza virus. *Influenza Other Respi Viruses,* 1, 113-120.

Bollback, J. P. & Huelsenbeck, J. P. 2001. Phylogeny, genome evolution, and host specificity of single-stranded RNA bacteriophage (family Leviviridae). *J Mol Evol,* 52, 117-128.

Brachman, P. S., Ehrlich, R., Maltman, M. F., Middlebrook, G., Morton, J. D., Silver, I. H. & Wolfe, E. K. 1964. Standard sampler for assay of airborne microorganisms. *Science,* 144, 1295.

Bridges, C. B., Kuehnert, M. J. & Hall, C. B. 2003. Transmission of influenza: implications for control in health care settings. *Clin Infect Dis,* 37, 1094-1101.

Brundage, J. F., Scott, R. M., Lednar, W. M., Smith, D. W. & Miller, R. N. 1988. Building-associated risk of febrile acute respiratory diseases in Army trainees. *Jama,* 259, 2108-12.

Bullough, P. A., Hughson, F. M., Skehel, J. J. & Wiley, D. C. 1994. Structure of influenza haemagglutinin at the pH of membrane fusion. *Nature,* 371, 37-43.

Burkdolder, H. C. & Berg, J. C. 1974. *AIChE J.,* 20, 872-880.

Carducci, A., Gemelli, C., Cantiani, L., Casini, B. & Rovini, E. 1999. Assessment of microbial parameters as indicators of viral contamination of aerosol from urban sewage treatment plants. *Lett Appl Microbiol,* 28, 207-210.

Cherian, T., Bobo, L., Steinhoff, M. C., Karron, R. A. & Yolken, R. H. 1994. Use of PCR-enzyme immunoassay for identification of influenza A virus matrix RNA in clinical samples negative for cultivable virus. *J Clin Microbiol,* 32, 623-628.

Claas, E. C., Osterhaus, A. D., van Beek, R., De Jong, J. C., Rimmelzwaan, G. F., Senne, D. A., Krauss, S., Shortridge, K. F. & Webster, R. G. 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. *Lancet,* 351, 472-477.

Collins, M. L., Zayati, C., Detmer, J. J., Daly, B., Kolberg, J. A., Cha, T. A., Irvine, B. D., Tucker, J. & Urdea, M. S. 1995. Preparation and characterization of RNA standards for use in quantitative branched DNA hybridization assays. *Anal Biochem,* 226, 120-129.

Couch, R. B., Kasel, J. A., Glezen, W. P., Cate, T. R., Six, H. R., Taber, L. H., Frank, A. L., Greenberg, S. B., Zahradnik, J. M. & Keitel, W. A. 1986. Influenza: its control in persons and populations. *J Infect Dis,* 153, 431-440.

Cox, C. S. & Wathes, C. M. 1995. Bioaerosols in the environment. *Bioaerosols handbook.* Boca Raton: CRC Press.

Daigle, C. C., Chalupa, D. C., Gibb, F. R., Morrow, P. E., Oberdorster, G., Utell, M. J. & Frampton, M. W. 2003. Ultrafine particle deposition in humans during rest and exercise. *Inhal Toxicol,* 15, 539-552.

Donofrio, J. C., Coonrod, J. D., Davidson, J. N. & Betts, R. F. 1992. Detection of influenza A and B in respiratory secretions with the polymerase chain reaction. *PCR Methods Appl,* 1, 263-268.

Echevarria, J. E., Erdman, D. D., Swierkosz, E. M., Holloway, B. P. & Anderson, L. J. 1998. Simultaneous detection and identification of human parainfluenza viruses 1, 2, and 3 from clinical samples by multiplex PCR. *J Clin Microbiol,* 36, 1388-1391.

Edelman, D. C. & Barletta, J. 2003. Real-time PCR provides improved detection and titer determination of bacteriophage. *Biotechniques,* 35, 368-375.

Eduard, W. & Heederik, D. 1998. Methods for quantitative assessment of airborne levels of noninfectious microorganisms in highly contaminated work environments. *Am Ind Hyg Assoc J,* 59, 113-127.

Edwards, D. A., Brenner, H. & Wasan, D. T. 1991. *Processes and Rheology.* Boston: Butterworth-Heinemann.

Edwards, D. A., Man, J. C., Brand, P., Katstra, J. P., Sommerer, K., Stone, H. A., Nardell, E. & Scheuch, G. 2004. Inhaling to mitigate exhaled bioaerosols. *Proc Natl Acad Sci U S A,* 101, 17383-17388.

Ellis, J. S. & Zambon, M. C. 2001. Combined PCR-heteroduplex mobility assay for detection and differentiation of influenza A viruses from different animal species. *J Clin Microbiol,* 39, 4097-102.

Erdal, S. & Esmen, N. A. 1995. Human head model as an aerosol sampler: Calculation of aspiration efficiencies for coarse particles using an idealized human head model facing the wind. *J Aerosol Sci,* 26, 253-272.

Fabian, P., McDevitt, J. J., DeHaan, W. H., Fung, R. O., Cowling, B. J., Chan, K. H., Leung, G. M. & Milton, D. K. 2008. Influenza virus in human exhaled breath: an observational study. *PLoS One,* 3, e2691.

Fabian, P., McDevitt, J. J., Houseman, E. A. & Milton, D. K. 2009a. Airborne influenza virus detection with four aerosol samplers using molecular and infectivity assays: considerations for a new infectious virus aerosol sampler. *Indoor Air,* 19, 433-441.

Fabian, P., McDevitt, J. J., Lee, W. M., Houseman, E. A. & Milton, D. K. 2009b. An optimized method to detect influenza virus and human rhinovirus from exhaled breath and the airborne environment. *J Environ Monit,* 11, 314-317.

Fiegel, J., Clarke, R. & Edwards, D. A. 2006. Airborne infectious disease and the suppression of pulmonary bioaerosols. *Drug Discov Today,* 11, 51-57.

Fiore, A. E., Shay, D. K., Broder, K., Iskander, J. K., Uyeki, T. M., Mootrey, G., Bresee, J. S. & Cox, N. S. 2008. Prevention and control of influenza: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2008. *MMWR Recomm Rep,* 57, 1-60.

Fisk, W. J. 2000. Health and productivity gains from better indoor environments and their relationship with building energy efficiency. *Ann Rev Energy Env,* 25, 537-566.

Foarde, K. K., Hanley, J. T., Ensor, D. S. & Roessler, P. 1999. Development of a method for measuring single-pass bioaerosol removal efficiencies of a room cleaner. *Aerosol Sci Tech,* 30, 223-234.

Gao, N. P. a. N., J.L. 2007. Modeling particle dispersion and deposition in indoor environments.

Garner, J. S. 1996. Guideline for isolation precautions in hospitals. Part I. Evolution of isolation practices, Hospital Infection Control Practices Advisory Committee. *Am J Infect Control,* 24, 24-31.

Gendron, L., Verreault, D., Veillette, M., Moineau, S. & Duchaine, C. 2010. Evaluation of Filters for the Sampling and Quantification of RNA Phage Aerosols. *Aerosol Science and Technology,* 44, 893-901.

Gerone, P. J., Couch, R. B. & Knight, V. 1971. Aerosol inoculator for exposure of human volunteers. *Appl Microbiol,* 22, 899-903.

Goldmann, D. A. 2000. Transmission of viral respiratory infections in the home. *Pediatr Infect Dis J,* 19, S97-S102.

Griffiths, W. D., Bennett, A., Speight, S. & Parks, S. 2005. Determining the performance of a commercial air purification system for reducing airborne contamination using model micro-organisms: a new test methodology. *J Hosp Infect,* 61, 242-247.

Griffiths, W. D. & Stewart, I. W. 1999. Performance of bioaerosol samplers used by the UK biotechnology industry. *J Aerosol Sci,* 30, 1029-1040.

Grinshpun, S. A., Chang, C.-W., Nevalainen, A. & Willeke, K. 1994. Inlet characteristics of bioaerosol samplers. *J Aerosol Sci,* 25, 1503-1522.

Grinshpun, S. A., Willeke, K., Ulevicius, V., Donnelly, J., Lin, X. & Mainelis, G. 1996. Collection of airborne microorganisms: advantages and disadvantages of different methods. *J Aerosol Sci,* 27, S247-S248.

Guerin, L. F. & Mitchell, C. A. 1964. A method for determining concentration of airborne virus and sizing droplet nuclei containing the agent. *Can J Comp Med Vet Sci,* 28, 283-287.

Hall, C. B. 2001. Respiratory syncytial virus and parainfluenza virus. *N Engl J Med,* 344, 1917-28.

Harper, G. J. 1961. Airborne micro-organisms: survival tests with four viruses. *J Hyg (Lond),* 59, 479-486.

Hatch, T. F. 1961. Distribution and deposition of inhaled particles in respiratory tract. *Bacteriol Rev,* 25, 237-240.

Heath, G. S., King, D. P., Turner, J. L. E., Wakeley, P. R. & Banks, M. 2003. Use of an internal standard in a TaqMan(R) nested reverse transcription-polymerase chain reaction for the detection of bovine viral diarrhoea virus. *Vet Microbiol,* 96, 357-366.

Heid, C. A., Stevens, J., Livak, K. J. & Williams, P. M. 1996. Real time quantitative PCR. *Genome Res,* 6, 986-994.

Hemmes, J. H., Winkler, K. C. & Kool, S. M. 1960. Virus survival as a seasonal factor in influenza and polimyelitis. *Nature,* 188, 430-431.

Hermann, J. R., Hoff, S. J., Yoon, K. J., Burkhardt, A. C., Evans, R. B. & Zimmerman, J. J. 2006. Optimization of a sampling system for recovery and detection of airborne porcine

reproductive and respiratory syndrome virus and swine influenza virus. *Appl Environ Microbiol,* 72, 4811-8.

Heyder, J. & Rudolf, G. Year. Deposition of aerosol particles in the human nose. *In:* WALTON, W. H., ed. Internation Symposium of The British Occupational Hygiene Society, 1977. Pergamon Press, 107-126.

Hindiyeh, M., Levy, V., Azar, R., Varsano, N., Regev, L., Shalev, Y., Grossman, Z. & Mendelson, E. 2005. Evaluation of a multiplex real-time reverse transcriptase PCR assay for detection and differentiation of influenza viruses A and B during the 2001-2002 influenza season in Israel. *J Clin Microbiol,* 43, 589-95.

Hinds, W. C. 1982. *Aerosol Technology: Properties, Behaviour and Measurement of Airborne Particles,* New York, John Wiley & Sons.

Hino, S. & Sato, H. 1984. Collison 6-jet nebulizer: choice for the certification test of the class II biological safety cabinet. *Jpn J Exp Med,* 54, 29-38.

Hogan, C. J., Jr., Kettleson, E. M., Lee, M. H., Ramaswami, B., Angenent, L. T. & Biswas, P. 2005. Sampling methodologies and dosage assessment techniques for submicrometre and ultrafine virus aerosol particles. *J Appl Microbiol,* 99, 1422-34.

Holland, J., Spindler, K., Horodyski, F., Grabau, E., Nichol, S. & VandePol, S. 1982. Rapid evolution of RNA genomes. *Science,* 215, 1577-85.

Holland, P. M., Abramson, R. D., Watson, R. & Gelfand, D. H. 1991. Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proc Natl Acad Sci U S A,* 88, 7276-80.

Hsieh, Y. C., Wu, T. Z., Liu, D. P., Shao, P. L., Chang, L. Y., Lu, C. Y., Lee, C. Y., Huang, F. Y. & Huang, L. M. 2006. Influenza pandemics: past, present and future. *J Formos Med Assoc,* 105, 1-6.

Ijaz, M. K., Karim, Y. G., Sattar, S. A. & Johnson-Lussenburg, C. M. 1987. Development of methods to study the survival of airborne viruses. *J Virol Methods,* 18, 87-106.

Ijaz, M. K., Sattar, S. A., Johnson-Lussenburg, C. M., Springthorpe, V. S. & Nair, R. C. 1985. Effect of relative humidity, atmospheric temperature, and suspending medium on the airborne survival of human rotavirus. *Can J Microbiol,* 31, 681-5.

Johnson, G. R. & Morawska, L. 2009. The mechanism of breath aerosol formation. *J Aerosol Med Pulm Drug Deliv,* 22, 229-37.

Kaczmarek, M., Owen, R. & Barr, I. G. 2010. Annual report of the National Influenza Surveillance Scheme. *Commun Dis Intell,* 34, 8-22.

Karim, Y. G., Ijaz, M. K., Sattar, S. A. & Johnson-Lussenburg, C. M. 1985. Effect of relative humidity on the airborne survival of rhinovirus-14. *Can J Microbiol,* 31, 1058-61.

Kenny, L. C., Bowry, A., Crook, B. & Stancliffe, J. D. 1999. Field testing of a personal size-selective bioaerosol sampler. *Ann Occup Hyg,* 43, 393-404.

King, M., Brock, G. & Lundell, C. 1985. Clearance of mucus by simulated cough. *J Appl Physiol,* 58, 1776-1782.

Knight, V. 1973. *Viral and Mycoplasmal Infections of the Respiratory Tract,* Philadelphia, Lea & Febiger.

Knight, V. 1980. Viruses as agents of airborne contagion. *Ann N Y Acad Sci,* 353, 147-56.

Korteweg, C. & Gu, J. 2008. Pathology, molecular biology, and pathogenesis of avian influenza A (H5N1) infection in humans. *Am J Pathol,* 172, 1155-70.

Kowalski, W. J. & Bahnfleth, W. 1998. Airborne respiratory diseases and mechanical systems for control of microbes. *Heating, Piping, and Air Conditioning,* 70, 34.

Lefeuvre, A., Contamin, H., Decelle, T., Fournier, C., Lang, J., Deubel, V. & Marianneau, P. 2006. Host-cell interaction of attenuated and wild-type strains of yellow fever virus can be differentiated at early stages of hepatocyte infection. *Microbes Infect,* 8, 1530-8.

Li, J., Hampson, A., Roche, P. W., Yohannes, K. & Spencer, J. D. 2005a. Annual report of the National Influenza Surveillance Scheme, 2004. *Commun Dis Intell,* 29, 125-36.

Li, Y., Duan, S., Yu, I. T. & Wong, T. W. 2005b. Multi-zone modeling of probable SARS virus transmission by airflow between flats in Block E, Amoy Gardens. *Indoor Air,* 15, 96-111.

Lin, X., A. Reponen, T., Willeke, K., Grinshpun, S. A., Foarde, K. K. & Ensor, D. S. 1999. Long-term sampling of airborne bacteria and fungi into a non-evaporating liquid. *Atmospheric Environment,* 33, 4291-4298.

Lindsley, W. G., Schmechel, D. & Chen, B. T. 2006. A two-stage cyclone using microcentrifuge tubes for personal bioaerosol sampling. *J Environ Monit,* 8, 1136-42.

Liolios, L., Jenney, A., Spelman, D., Kotsimbos, T., Catton, M. & Wesselingh, S. 2001. Comparison of a multiplex reverse transcription-PCR-enzyme hybridization assay with conventional viral culture and immunofluorescence techniques for the detection of seven viral respiratory pathogens. *J Clin Microbiol,* 39, 2779-2783.

Loosli, C. G., Lemon, H.M., Robertson, O.H., Appel, E. 1943. Experimental airborne influenza infection. I. Influence of humidity on survival of virus in air. *Proc Soc Exp Biol Med,* 53, 205-206.

Loudon, R. G. & Roberts, R. M. 1967. Droplet expulsion from the respiratory tract. *Am Rev Respir Dis,* 95, 435-42.

Lynch, J. P., 3rd & Walsh, E. E. 2007. Influenza: evolving strategies in treatment and prevention. *Semin Respir Crit Care Med,* 28, 144-58.

Macher, J. M. 1989. Positive-Hole Correction of Multiple-Jet Impactors for Collecting Viable Microorganisms. *Am Ind Hyg Assoc J,* 50, 561 - 568.

Mackay, I. M., Arden, K. E. & Nitsche, A. 2002. Real-time PCR in virology. *Nucleic Acids Res,* 30, 1292-305.

Magnard, C., Valette, M., Aymard, M. & Lina, B. 1999. Comparison of two nested PCR, cell culture, and antigen detection for the diagnosis of upper respiratory tract infections due to influenza viruses. *J Med Virol,* 59, 215-220.

May, K. R. 1973. The collison nebulizer: Description, performance and application. *J Aerosol Sci,* 4, 235-238, IN1, 239-243.

McCluskey, R., Sandin, R. & Greene, J. 1996. Detection of airborne cytomegalovirus in hospital rooms of immuncompromised patients. *J Virol Methods,* 56, 115-118.

McGoldrick, A., Lowings, J. P., Ibata, G., Sands, J. J., Belak, S. & Paton, D. J. 1998. A novel approach to the detection of classical swine fever virus by RT-PCR with a fluorogenic probe (TaqMan). *J Virol Methods,* 72, 125-135.

Mitchell, C. A. & Guerin, L. F. 1972. Influenza A of human, swine, equine and avian origin: comparison of survival in aerosol form. *Can J Comp Med,* 36, 9-11.

Mitchell, C. A., Guerin, L. F. & Robillard, J. 1968. Decay of influenza A viruses of human and avian origin. *Can J Comp Med,* 32, 544-6.

Morawska, L. 2006. Droplet fate in indoor environments, or can we prevent the spread of infection? *Indoor Air,* 16, 335-47.

Morawska, L., Johnson, G., Ristovski, Z., Hargreaves, M., Mengersen, K. L., Corbett, S., Chao, C., Li, Y. & Katoshevski, D. 2009. Size distribution and sites of origin of droplets expelled during expiratory activities. *J Aerosol Sci,* 40, 256-269.

Morawska, L., Johnson, G. R., Ristovski, Z., Hargreaves, M., Mengersen, K. L., Chao, C., Wan, M. P., Li, Y., Xie, X. & Katoshevski, D. 2008. Droplets expelled during human expiratory activities and their origin. *11th International Conference on Indoor Air Quality and Climate.*

Murphy, B. R., Baron, S., Chalhub, E. G., Uhlendorf, C. P. & Chanock, R. M. 1973. Temperature-Sensitive Mutants of Influenza Virus. IV. Induction of Interferon in the

Nasopharynx by Wild-Type and a Temperature-Sensitive Recombinant Virus. *J Infect Dis,* 128, 488-493.

Myatt, T. A., Johnston, S. L., Rudnick, S. & Milton, D. K. 2003. Airborne rhinovirus detection and effect of ultraviolet irradiation on detection by a semi-nested RT-PCR assay. *BMC Public Health,* 3, 5.

Nelson, D. 2004. Phage taxonomy: we agree to disagree. *J Bacteriol,* 186, 7029-31.

Nicas, M., Nazaroff, W. W. & Hubbard, A. 2005. Toward understanding the risk of secondary airborne infection: emission of respirable pathogens. *J Occup Environ Hyg,* 2, 143-54.

Nicholls, J. M., Chan, R. W., Russell, R. J., Air, G. M. & Peiris, J. S. 2008. Evolving complexities of influenza virus and its receptors. *Trends Microbiol,* 16, 149-57.

O'Connell, K. P., Bucher, J. R., Anderson, P. E., Cao, C. J., Khan, A. S., Gostomski, M. V. & Valdes, J. J. 2006. Real-time fluorogenic reverse transcription-PCR assays for detection of bacteriophage MS2. *Appl Environ Microbiol,* 72, 478-83.

Olson, M. R., Axler, R. P. & Hicks, R. E. 2004. Effects of freezing and storage temperature on MS2 viability. *J Virol Methods,* 122, 147-52.

Pace, N. R. 1997. A molecular view of microbial diversity and the biosphere. *Science,* 276, 734-40.

Papineni, R. S. & Rosenthal, F. S. 1997. The size distribution of droplets in the exhaled breath of healthy human subjects. *J Aerosol Med,* 10, 105-16.

Peccia, J. & Hernandez, M. 2006. Incorporating polymerase chain reaction-based identification, population characterization, and quantification of microorganisms into aerosol science: A review. *Atmos Environ,* 40, 3941-3961.

Perkins, S. M., Webb, D. L., Torrance, S. A., El Saleeby, C., Harrison, L. M., Aitken, J. A., Patel, A. & DeVincenzo, J. P. 2005. Comparison of a real-time reverse transcriptase PCR assay and a culture technique for quantitative assessment of viral load in children naturally infected with respiratory syncytial virus. *J Clin Microbiol,* 43, 2356-2362.

Petrik, J., Pearson, G. J. M. & Allain, J.-P. 1997. High throughput PCR detection of HCV based on semiautomated multisample RNA capture. *J Virol Methods,* 64, 147-159.

Pillai, S. D. 2002. Bioaerosols and public health. *In:* INSAM, H., RIDDECH, N. & KLAMMER, S. (eds.) *Microbiology of composting.* Berlin: Springer-Verlag GmbH & Co.

Pillai, S. D. & Ricke, S. C. 2002. Bioaerosols from municipal and animal wastes: background and contemporary issues. *Can J Microbiol,* 48, 681-96.

Proctor, D. F. 1966. Airborne disease and the upper respiratory tract. *Bacteriol Rev,* 30, 498-513.

Rose, J. B., Zhou, X., Griffin, D. W. & Paul, J. H. 1997. Comparison of PCR and Plaque Assay for Detection and Enumeration of Coliphage in Polluted Marine Waters. *Appl Environ Microbiol,* 63, 4564-4566.

Russell, M. L., Goth-Goldstein, R., Apte, M. G. & Fisk, W. J. 2002. *Method for measuring the size distribution of airborne rhinovirus.* University of California.

Sattar, S. A. & Ijaz, M. K. 1997. Airborne Viruses. *In:* STETZENBACH, L. (ed.) *Manual of Environmental Microbiology.* Washington, USA: ASM Press.

Scarpino, P. V., Jensen, N. J., Jensen, P. A. & Ward, R. 1998. The use of ultraviolet germicidal irradiation (UVGI) in disinfection of airborne bacteria and rhinoviruses. *J Aerosol Sci*

Proceedings of the 1998 International Aerosol Conference Part 2, 29, S777-S778.

Schaffer, F. L., Soergel, M. E. & Straube, D. C. 1976. Survival of airborne influenza virus: effects of propagating host, relative humidity, and composition of spray fluids. *Arch Virol,* 51, 263-73.

Schweiger, B., Zadow, I., Heckler, R., Timm, H. & Pauli, G. 2000. Application of a fluorogenic PCR assay for typing and subtyping of influenza viruses in respiratory samples. *J Clin Microbiol,* 38, 1552-1558.

Schweigkofler, W., O'Donnell, K. & Garbelotto, M. 2004. Detection and quantification of airborne conidia of Fusarium circinatum, the causal agent of pine pitch canker, from two California sites by using a real-time PCR approach combined with a simple spore trapping method. *Appl Environ Microbiol,* 70, 3512-3520.

Sedlmaier, N., Hoppenheidt, K., Krist, H., Lehmann, S., Lang, H. & Buttner, M. 2009. Generation of avian influenza virus (AIV) contaminated fecal fine particulate matter (PM(2.5)): genome and infectivity detection and calculation of immission. *Vet Microbiol,* 139, 156-164.

Shinya, K., Ebina, M., Yamada, S., Ono, M., Kasai, N. & Kawaoka, Y. 2006. Avian flu: influenza virus receptors in the human airway. *Nature,* 440, 435-436.

Smith, G., Smith, I., Harrower, B., Warrilow, D. & Bletchly, C. 2006. A simple method for preparing synthetic controls for conventional and real-time PCR for the identification of endemic and exotic disease agents. *J Virol Methods,* 135, 229-234.

Sobsey, M. D. & Meschke, J. S. 2003. Virus survival in the environment with special attention to survival in sewage droplets and other environmental media of fecal or respiratory origin. International association of plumbing and mechanical officials.

Stewart, J. J. 2002. Parameters of influenza aerosol transmission. *Comments on Theoretical Biology,* 7, 445-457.

Stockton, J., Ellis, J. S., Saville, M., Clewley, J. P. & Zambon, M. C. 1998. Multiplex PCR for typing and subtyping influenza and respiratory syncytial viruses. *J Clin Microbiol,* 36, 2990-2995.

Stram, Y., Kuznetzova, L., Guini, M., Rogel, A., Meirom, R., Chai, D., Yadin, H. & Brenner, J. 2004. Detection and quantitation of akabane and aino viruses by multiplex real-time reverse-transcriptase PCR. *J Virol Methods,* 116, 147-154.

Tang, J. W., Li, Y., Eames, I., Chan, P. K. S. & Ridgway, G. L. 2006. Factors involved in the aerosol transmission of infection and control of ventilation in healthcare premises. *J Hosp Infect,* 64, 100-114.

Tanner, B. D., Brooks, J. P., Haas, C. N., Gerba, C. P. & Pepper, I. L. 2005. Bioaerosol emission rate and plume characteristics during land application of liquid class B biosolids. *Environ Sci Technol,* 39, 1584-1590.

Tellier, R. 2006. Review of Aerosol Transmission of Influenza A Virus. *Emerg Infect Dis,* 12, 1657-1662.

Thompson, S. S. & Yates, M. V. 1999. Bacteriophage inactivation at the air-water-solid interface in dynamic batch systems. *Appl Environ Microbiol,* 65, 1186-1190.

Tseng, C. C. & Li, C. S. 2005. Collection efficiencies of aerosol samplers for virus-containing aerosols. *J Aerosol Sci,* 36, 593-607.

Upton, S. L., Mark, D., Douglass, E. J., Hall, D. J. & Griffiths, W. D. 1994. A wind tunnel evaluation of the physical sampling efficiencies of three bioaerosol samplers. *J Aerosol Sci,* 25, 1493-1501.

Utrup, L. J. & Frey, A. H. 2004. Fate of bioterrorism-relevant viruses and bacteria, including spores, aerosolized into an indoor air environment. *Exp Biol Med (Maywood),* 229, 345-350.

Van Cuyk, S., Siegrist, R. L., Lowe, K. & Harvey, R. W. 2004. Evaluating microbial purification during soil treatment of wastewater with multicomponent tracer and surrogate tests. *J Environ Qual,* 33, 316-329.

van Elden, L. J., Nijhuis, M., Schipper, P., Schuurman, R. & van Loon, A. M. 2001. Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. *J Clin Microbiol,* 39, 196-200.

van Riel, D., den Bakker, M. A., Leijten, L. M., Chutinimitkul, S., Munster, V. J., de Wit, E., Rimmelzwaan, G. F., Fouchier, R. A., Osterhaus, A. D. & Kuiken, T. 2010. Seasonal

and pandemic human influenza viruses attach better to human upper respiratory tract epithelium than avian influenza viruses. *Am J Pathol,* 176, 1614-1618.

Vincent, J. H. 1989. *Aerosol sampling: Science and Practice,* Chichester, John Wiley & Sons.

Wang, B., Zhang, A., Sun, J. L., Liu, H., Hu, J. & Xu, L. X. 2005. Study of SARS transmission via liquid droplets in air. *J Biomech Eng,* 127, 32-38.

Weber, T. P. & Stilianakis, N. I. 2008. Inactivation of influenza A viruses in the environment and modes of transmission: a critical review. *J Infect,* 57, 361-373.

Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M. & Kawaoka, Y. 1992. Evolution and ecology of influenza A viruses. *Microbiol Rev,* 56, 152-179.

Wiley, D. C. & Skehel, J. J. 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu Rev Biochem,* 56, 365-394.

Wilson, S. C., Morrow-Tesch, J., Straus, D. C., Cooley, J. D., Wong, W. C., Mitlohner, F. M. & McGlone, J. J. 2002. Airborne microbial flora in a cattle feedlot. *Appl Environ Microbiol,* 68, 3238-3242.

World Health Organisation. 2006. *Avian influenza ("bird flu") - fact sheet* [Online]. World Health Organisation. Available: http://www.who.int/csr/disease/avian_influenza/avianinfluenza_factsheetJan2006/en/index.html [Accessed February 5 2011].

World Health Organisation. 2009. *Influenza (seasonal)* [Online]. World Health Organisation. Available: http://www.who.int/mediacentre/factsheets/fs211/en/index.html [Accessed 10 November 2010].

World Health Organisation. 2010a. *Avian Influenza* [Online]. Available: http://www.who.int/mediacentre/factsheets/avian_influenza/en/index.html [Accessed 23 November 2010].

World Health Organisation. 2010b. *Influenza* [Online]. Copenhagen: WHO Regional Office for Europe. Available: http://www.euro.who.int/en/what-we-do/health-topics/diseases-and-conditions/influenza/seasonal-influenza [Accessed November 23 2010].

World Health Organization. 2010. *Pandemic (H1N1) 2009* [Online]. World Health Organization. Available: http://www.who.int/csr/disease/swineflu/en/index.html [Accessed February 6 2011].

Wu, Q., Xu, Z., Wei, T., Zeng, H., Li, J., Gang, H., Sun, M., Jiang, F., Wang, X. & Dong, W. 2004. Development of Taqman RT-nested PCR system for clinical SARS-CoV detection. *J Virol Methods,* 119, 17-23.

Yohannes, K., Roche, P., Hampson, A., Miller, M. & Spencer, J. 2004. Annual report of the National Influenza Surveillance Scheme, 2003. *Commun Dis Intell,* 28, 160-168.

Yu, I. T., Li, Y., Wong, T. W., Tam, W., Chan, A. T., Lee, J. H., Leung, D. Y. & Ho, T. 2004. Evidence of airborne transmission of the severe acute respiratory syndrome virus. *N Engl J Med,* 350, 1731-9.

Yuen, K. Y., Chan, P. K., Peiris, M., Tsang, D. N., Que, T. L., Shortridge, K. F., Cheung, P. T., To, W. K., Ho, E. T., Sung, R. & Cheng, A. F. 1998. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. *Lancet,* 351, 467-471.

Zeng, Q. Y., Westermark, S. O., Rasmuson-Lestander, A. & Wang, X. R. 2004. Detection and quantification of Wallemia sebi in aerosols by real-time PCR, conventional PCR, and cultivation. *Appl Environ Microbiol,* 70, 7295-7302.

Zhang, W. D. & Evans, D. H. 1991. Detection and identification of human influenza viruses by the polymerase chain reaction. *J Virol Methods,* 33, 165-189.

Appendix A: Publication (Chapter 3)

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

A nested real-time PCR assay has an increased sensitivity suitable for detection of viruses in aerosol studies

P. Perrott[1,2], G. Smith[3], Z. Ristovski[2], R. Harding[1] and M. Hargreaves[1,2]

1 School of Life Sciences, Queensland University of Technology, Brisbane, Qld, Australia
2 International Laboratory of Air Quality and Health, QUT, Brisbane, Qld, Australia
3 Virology, Queensland Health Forensic and Scientific Services, Brisbane, Qld, Australia

Appendix B: Synthetic Control Sequences

MS2 synthetic oligonucleotides

Forward strand

F1

5' GGTAATACGACTCACTATAGGGGGGGTGAACAAGCAACCGTTACCCCCCGCGCTCTGAGAGCGGCTCTATTGG **3'**

F2

5' TCCGAGACCAATGTGCGCCGTGGATCAGACACGCGGTCCGCTATAACGAGTCATATGAATTTAGGCTCGTT **3'**

F3

5' GTAGGGAACGGAGTGTTTACAGTTCCGAAGAATAATAAAATAGATCGGGCTGCCTGTAAGGAGCCTGATA **3'**

Reverse strand

R1

5' TATCAGGCTCCTTACAGGCAGCCCGATCTATTTTATTATTCTTCGGAACTGTAAACACTCCGTTCCCTACAACGAG **3'**

R2

5' CCTAAATTCATATGACTCGTTATAGCGGACCGCGTGTCTGATCCACGGCGCACATTGGTCTCGGACCAATA **3'**

R3

5' GAGCCGCTCTCAGAGCGCGGGGGGTAACGGTTGCTTGTTCACCCCCCCTATAGTGAGTCGTATTACC **3'**

MS2 primers for synthetic control

Forward

5' GGTAATACGACTCACTATAGGGGGGG **3'**

Reverse

5' TATCAGGCTCCTTACAGGCAGCC **3'**

Influenza synthetic oligonucleotides

Forward strand

F1

5' GGTAATACGACTCACTATAGGGGGGTCGCGCAGAGACTTGAAGATGTATTTGCTGGAAAGAATACCGATC **3'**

F2

5' TTGAGGCTCTCATGGAGTGGCTAAAGACAAGACCAATCCTGTCACCTCTGACTAAGGGGATTTTGGGGTTTGTGTTCACG **3'**

Reverse Strand

R1

5' CGTGAACACAAACCCCAAAATCCCCTTAGTCAGAGGTGACAGGATTGGTCTTGTCTTTAGCCACTCCA **3'**

R2

5' TGAGAGCCTCAAGATCGGTATTCTTTCCAGCAAATACATCTTCAAGTCTCTGCGCGACCCCCCTATAGTGAGTCGTATTACC **3'**

Influenza primers

Forward

5' GGTAATACGACTCACTATAGGGGGGTCG **3'**

Reverse

5' CGTGAACACAAACCCCAAAATCC **3'**

Key:

Yellow: T7 promoter sequence

Purple: extra bases to preserve target sequence