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Detection of Bacteriophage in Droplets

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1. Introduction

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. Acute respiratory diseases can be more severe for the young, elderly or immuno-compromised, causing complications and death in many cases.

Transmission of respiratory diseases, including of respiratory syncytial virus (RSV), can be via small droplets that are able to remain suspended in the air and subsequently be inhaled by a susceptible host (Centers for Disease Control, 2010). However, little information is available on the specifics of transmission via aerosol droplets. 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 study addresses some questions surrounding aspects of virus transmission via the aerosol route, in order to contribute to a more comprehensive understanding of this issue.

1.1 Transmission of viral infections

Respiratory infections can 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, also known as bioaerosols, 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 survival 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.

Respiratory syncytial virus (RSV) is a medically important virus, and is one of the two major causes of lower respiratory tract (LRT) infections in infants and young children. Recently, it has also been recognised as important pathogens in adults, although the symptoms are less distinct and often the viral cause is not initially correctly identified (Hall, 2001). Additionally, there are no effective means of controlling the spread of this virus.

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 alone. 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).

Of the recognised mechanisms by which respiratory viruses can spread, some consider 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 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 themselves can be pathogenic, 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, 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, agreement between 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 in the spread of respiratory infections like RSV. 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 as 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).

1.2.1 Bioaerosol production

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. 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. Activities such as coughing produce droplets which are much smaller, and in greater numbers, usually from the lower sections of the respiratory tract. Droplets produced from coughing and sneezing have been reported to range in size from 1 to 100 μm by one study (Kowalski and Bahnfleth, 1998) and from 1 to 20 μm by 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 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.

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).

1.2.2 Bioaerosol transport

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 respiratory viruses 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 study only investigated dynamic evaporation in the order of seconds, and the findings do 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, inertial forces and relative humidity (Pillai and Rieke, 2002). 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 Rieke, 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 regarding 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 $<1 \mu\text{m}$; 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 produced by infected people.

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-enveloped viruses tend to have a greater stability at lower RH, usually below 40 % (Benbough, 1971, Pillai and Ricke, 2002).

A review by Fiegel and co-workers (2006) concluded 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.

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. This review concluded that personal protective equipment is needed to prevent person-to-person short-range transmission of infectious diseases.

1.2.3 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 5 μm ; 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 particles 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).

Hinds (1982) described the fate of aerosol particles deposited in the respiratory tract as follows: 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 bind to the cells; and the immunological status of the individual.

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, a more thorough understanding is required of both virus persistence in droplets and the physical factors which significantly affect it.

1.3 Methods to study bioaerosol transport

Bacteriophages have played a key role in various types of research. A particularly 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. Bacteriophages are viruses that infect bacteria, and are thus are not harmful to humans. 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). 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).

Surrogate bacteriophages are selected based on a simple set of general criteria. 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 survival). MS2 is a commonly used bacteriophage in surrogate studies, including some studies investigating virus 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, 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. Furthermore, it has been used to compare the efficiencies of common air samplers (Tseng and Li, 2005).

An increasing number of studies have investigated the detection and/or survival of viruses in droplets; however extensive study of the persistence of respiratory viruses in aerosols has not been undertaken, nor has it been performed on the MS2 which is often used as a surrogate. Of particular interest is the quantification of virus survival when limiting factors are applied. As so few studies have been conducted regarding the actual aerosol characteristics of MS2 or common respiratory viruses, 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.

1.4 Studies of aerosolised virus

From previous simulated droplet experiments, it is known that virus survival 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 quite satisfactorily, they were not examined in this study. Instead, other limiting physical factors were investigated.

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.

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. 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. 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.

2. Study of MS2 bacteriophage and respiratory viruses in aerosols

The objective of our research was to develop a PCR assay to detect viruses in aerosols, and to characterise the fate of bacteriophage MS2 in aerosols produced from four different aerosol types, whilst varying physical factors.

2.1 Development of PCR assays to study viruses in aerosols

Respiratory viruses can be 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 assays for respiratory syncytial virus (RSV) and MS2 bacteriophage, which are sufficiently sensitive to be used in aerosol studies. To achieve this, a two-step, nested real-time PCR assay was developed. 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. Assays were also described for influenza A (H1N1 and H3N3), influenza B, and parainfluenza virus 1, and are described in Perrott et. al (2009).

2.1.1 Methods

Primer sets for the nested real-time PCR assays were designed based on previously described TaqMan® assays for each virus (see table 1). Using Primer Express®, an outer primer pair was designed to nest each TaqMan® primer/probe set. The outer primer pairs were 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.

The first step of the assay was a conventional PCR and was performed using SuperScript™ III One-Step RT-PCR with Platinum® Taq DNA polymerase mastermix (Invitrogen, Vic., Australia). For the second step (TaqMan® assay), Universal mix (Applied Biosystems) was prepared by adding primers and probe to the reaction mix. The assays were performed on an Applied Biosystems ABI 7500 System, and results are reported in cycle threshold (Ct) values.

| Organism | Reference | Primer set | Primer name | Sequence (5' -3') |
|-----------------------------------|------------------------|------------|-------------|----------------------------------|
| Human Respiratory Syncytial Virus | This study | Outer | RSV-F1 | TATTGTCATCGCCTTACAGTC |
| | | | RSV-R1 | CTAAGGCCAAAGCTTATACAG |
| | | Inner | RSVF | AGTAGACCATGTGAATTCCCTGC |
| | | | RSVR | GTCGATATCTTCATCACCATACTTTTCIGTTA |
| | | Probe | RSV probe | TCAATACCAGCTTATAGAAC |
| MS2 | This study | Outer | MS2-F5O | TGA ACA AGC AAC CGT TAC CCC |
| | | | MS2-R5O | TAT CAG GCT CCT TAC AGG CAG C |
| | O'Connell et al., 2006 | Inner | MS2F5 | GCT CTG AGA GCG GCT CTA TTG |
| | | | MS2R5 | CGT TAT AGC GGA CCG CGT |
| | | Probe | MS2-5 probe | CCGAGACCAATGTGCGCCGTG |

Table 1. All primers and probes 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.

2.1.2 Results and discussion

The real-time PCR assay for RSV detected the virus with a titre of 10^4 TCID₅₀ at 25.64 Ct, with an endpoint of 10 TCID₅₀, detected at 36.06 Ct (see figure 1). The nested real-time PCR assay detected the 10^4 TCID₅₀ 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 TCID₅₀, 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 was 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.

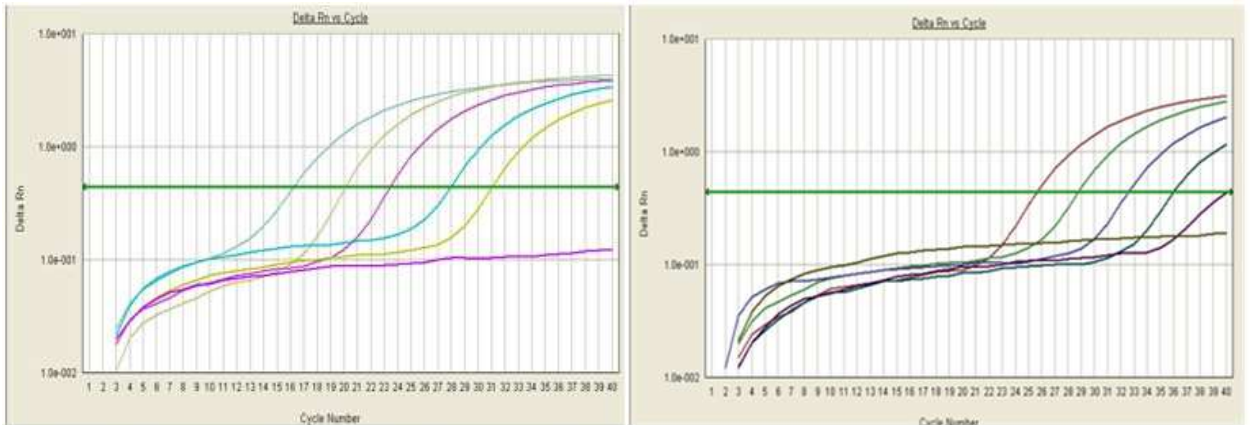


Fig. 1. RSV as detected by real-time PCR (left) and nested real-time PCR (right)

| Virus titre (TCID ₅₀) | | 10 ⁵ | 10 ⁴ | 10 ³ | 10 ² | 10 | 1 | 0.1 |
|-----------------------------------|-----------|-----------------|-----------------|-----------------|-----------------|----------|----------|----------|
| 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 |

Table 2. This table shows the results of the real-time and nested real-time PCR assays for detection of RSV and MS2 at several concentrations. The results displayed are cycle threshold values.

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.

Both the real-time PCR assay for MS2 and the nested real-time PCR assay for MS2 were used to detect MS2 bacteriophages from samples collected by gravitational settling, in order to determine which assay was more sensitive. 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 2. All amplified samples were detected from 8.65 to 37.73 Cts.

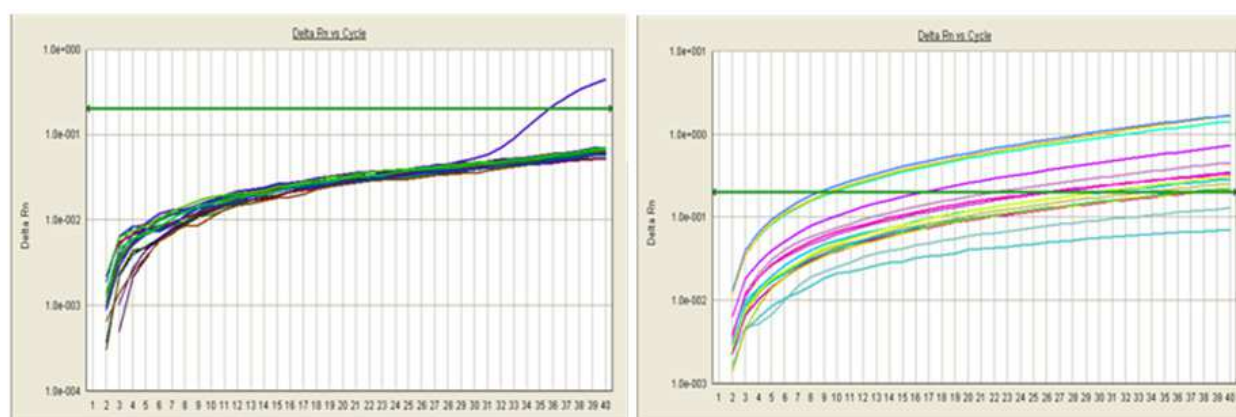


Fig. 2. The application of the real-time assay (left) and the nested real-time assay (right) to air samples

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.

Quantification of MS2 bacteriophage in aerosol samples using this method is described in the following section.

2.2 Bacteriophage in aerosols

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.

2.2.1 Methods

A Perspex chamber of approximately 150 L was used for all air testing experiments. Aerosols were created and delivered into the chamber with the Collison six-jet nebuliser (BGI Inc, Waltham, US), operated at a flow rate of six litres per minute (L/min), the air flow required to produce aerosols with a size of around 2 µm. Aerosol collection was performed using a six stage Andersen impactor. A collection plate with an *E. coli* overlay was placed into each of the six stages and each experiment was performed in triplicate. Plaques were counted the following day, and the positive-hole correction method was applied. Average (mean) counts were then calculated for each experiment.

We explored the following factors: concentration of virus in the nebulising suspension (10^5 to 10^3 PFU/mL); elapsed time between aerosolisation and sampling (0, 2, 5, 10 and 20 minutes); and the type of droplets ('wet droplets' were delivered directly into the chamber from the Collison, and 'dry droplets' which were mixed with sterile air at about 12 L/min, in order to dry the droplets before they were delivered into the chamber). Aerosolisation was performed for 30 seconds for each experiment.

Two types of nebulising suspension were used: traditionally-used phosphate buffered saline (PBS) and an artificial mucous (0.4 %), as described by King et. al (1985). Theoretically, the artificial mucous simulates an infected persons respiratory secretions more accurately than a water-like, non-viscous fluid. Thus, four droplet types were tested.

2.2.2 Results and discussion

2.2.2.1 Effect of droplet type

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 3). The droplets with the largest diameter were the wet droplets generated from PBS, with a mode of 0.980 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.

| 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 |

Table 3. Size distributions and droplet concentration of the four types of droplets used in this study, as measured using the UV-APS

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 cm³ 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.

2.2.2.2 Effect of elapsed time on MS2 as detected by the plaque assay and the PCR assay

When MS2 was aerosolised in PBS 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 ($\pm 780,684$) 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 ($\pm 381,113$) 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 ($\pm 274,203$) 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 ($\pm 174,191$) RNA copies detected; and at the last time point of 20 minutes elapsed time, the virus recovery rate rose slightly with 267,856 ($\pm 490,294$) RNA copies detected (0.38 %).

When MS2 was aerosolised from artificial mucous droplets, the nested real-time PCR assay was able to detect more relative RNA copies than infectious particles when collected after 20 minutes elapsed time. 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 PBS 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 ($\pm 6,859$) 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 ($\pm 7,281$) 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 ($\pm 1,751$) RNA copies detected.

When aerosolised in artificial mucous 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 elapsed 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.

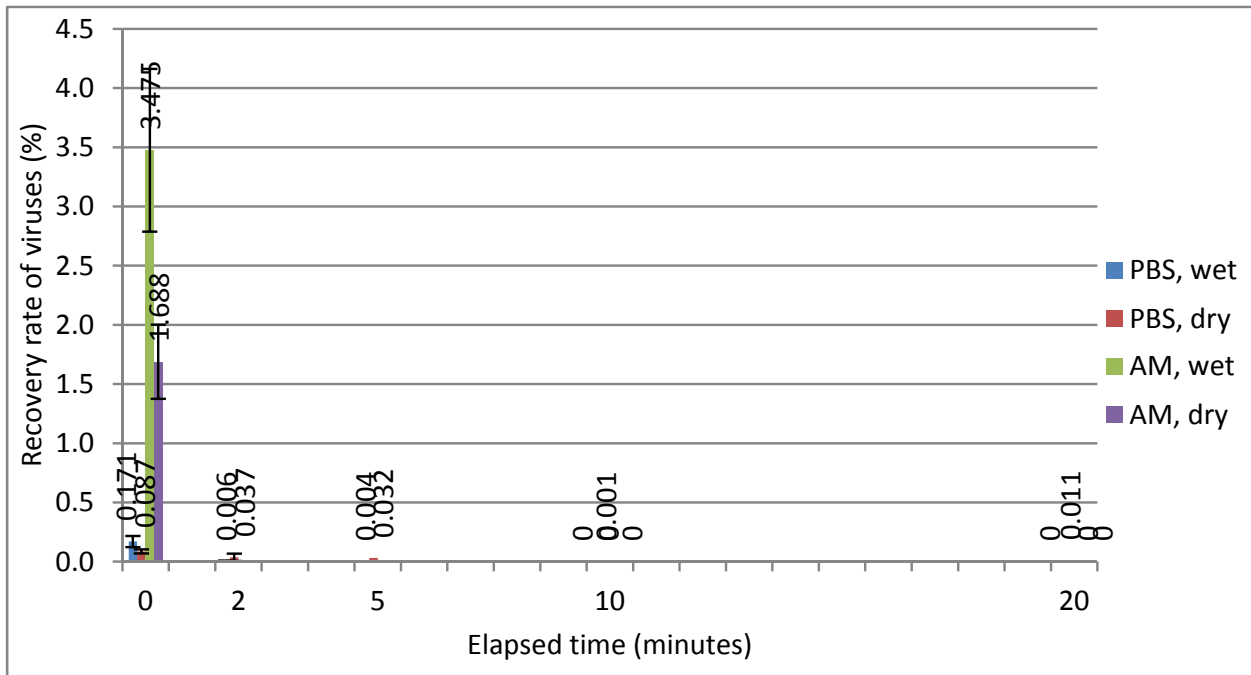


Fig. 3. The relative recovery of MS2 from the four different droplet types. Recoveries have been normalised to an initial challenge of 10⁵ PFU, collected after different elapsed times of zero minutes, two minutes, five minutes and ten minutes

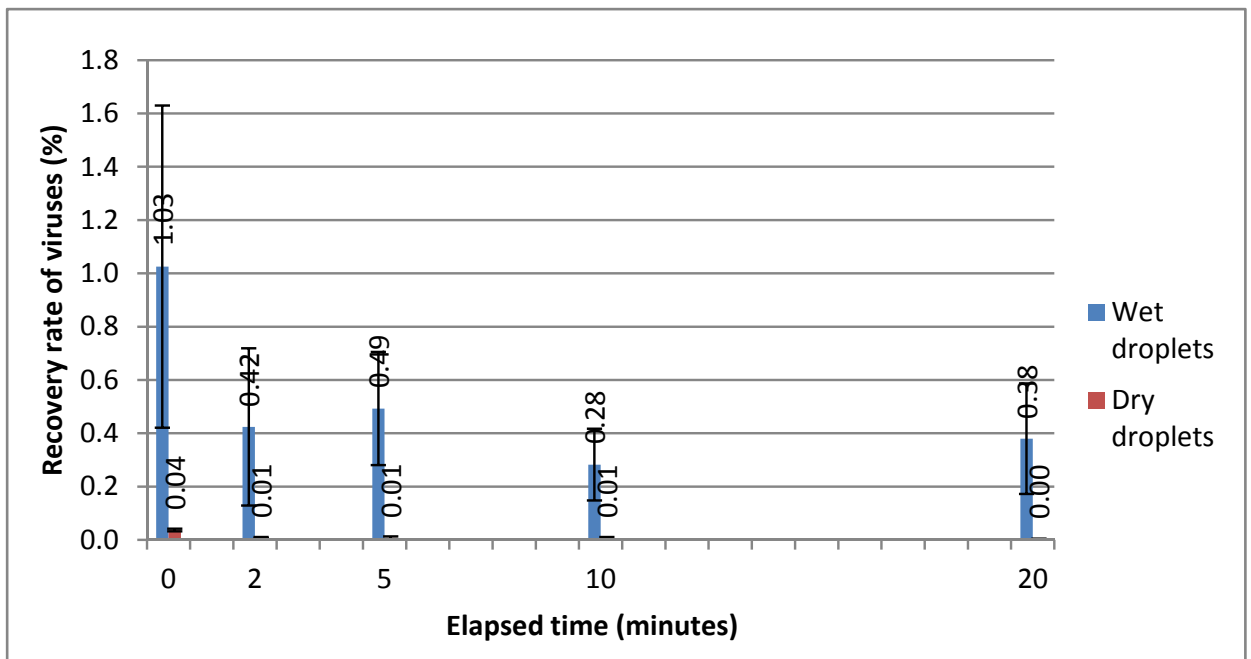


Fig. 4. Recovery of MS2 RNA copies from droplets generated from a PBS nebulising suspension and collected after varying elapsed times, detected by the nested real-time PCR assay

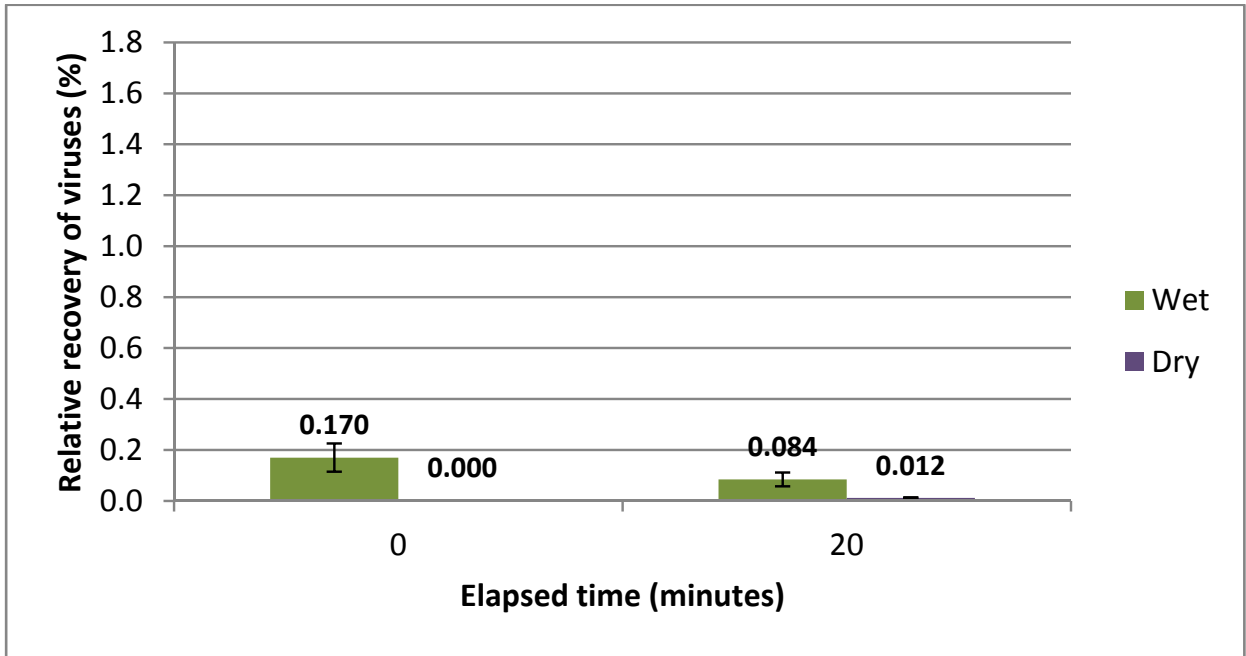


Fig. 5. Recovery of MS2 RNA copies from droplets generated from an artificial mucous suspension after varying elapsed times, as detected by the nested real-time PCR assay

A possible 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 an experiment examining this at different temperatures and times. 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 broth (used to propagate MS2 bacteriophage) 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.

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.

2.2.2.3 Effect of virus concentration in the nebulising suspension

For the PBS droplets, 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 and 7). From a challenge of 10⁵ PFU (equivalent to 1.3 x 10⁸ RNA copies), an average of 1,324,558 (± 780,684) RNA copies were recovered (1.03 %) from wet droplets. A challenge of 10⁴ PFU (1.3 x 10⁷ 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 10³ PFU (1.3 x 10⁶ 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 (\pm 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 (\pm 3193) RNA copies detected by the nested real-time PCR assay. No RNA was detected from the 10^3 PFU challenge.

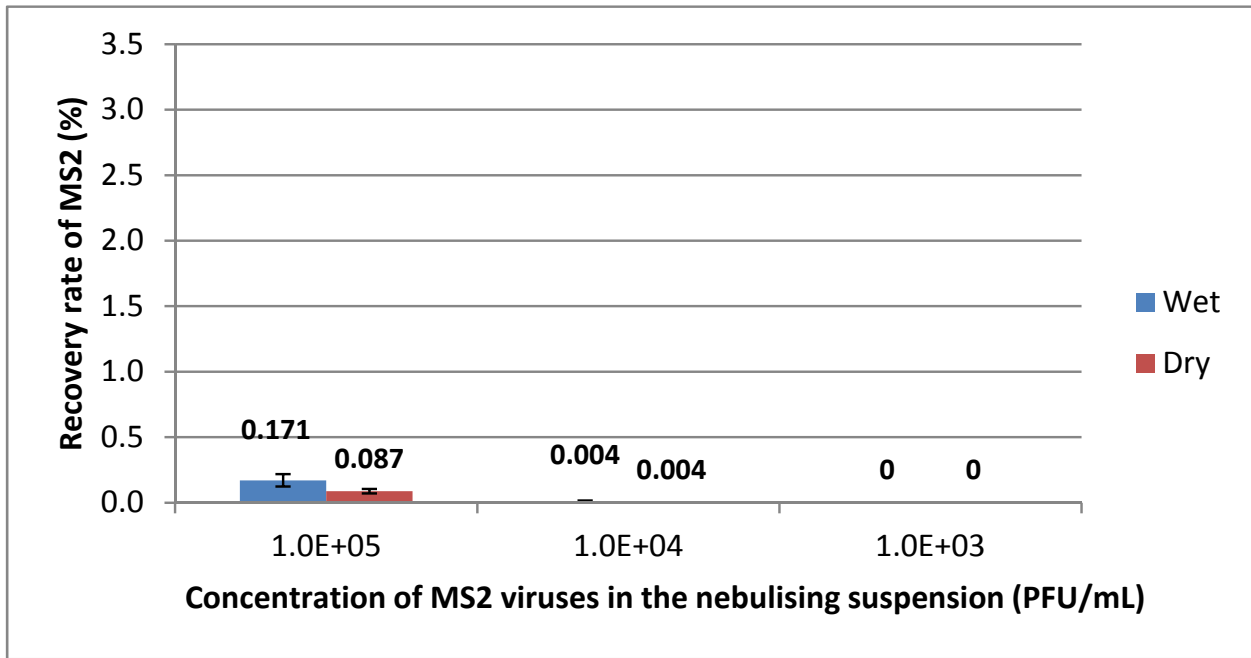


Fig. 6. Recovery of infectious MS2 from droplets generated from a PBS nebulising suspension of varying MS2 concentrations, as detected by the plaque assay

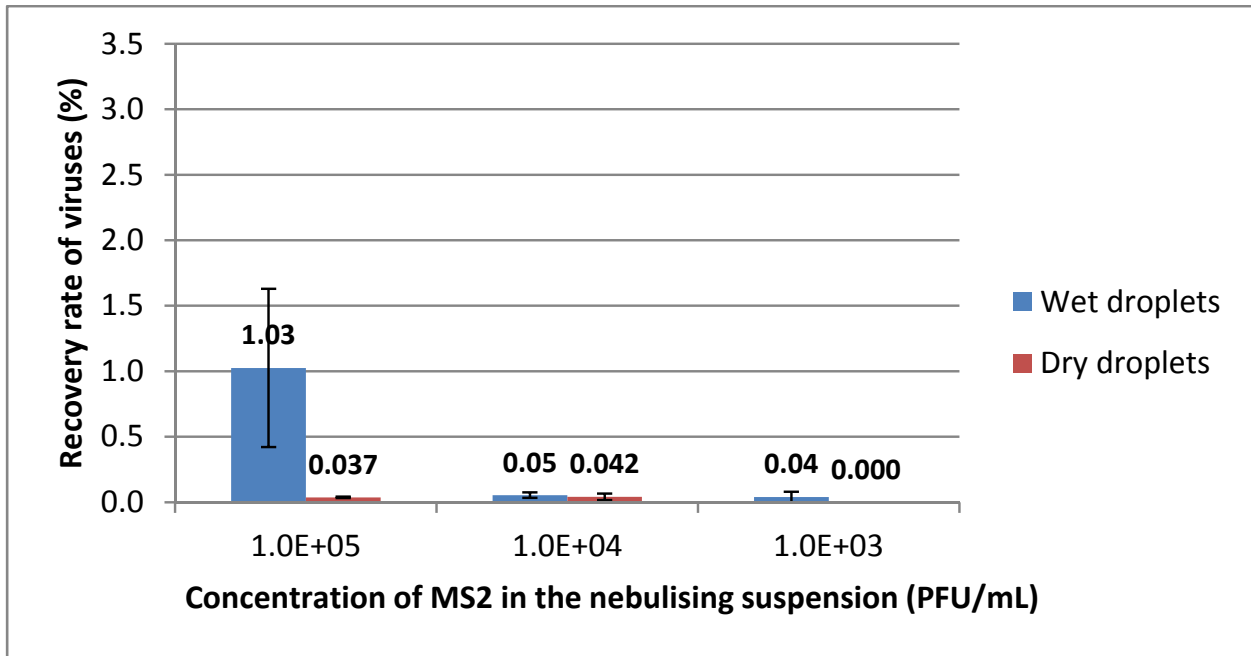


Fig. 7. Recovery of MS2 RNA copies from aerosols generated from PBS nebulising suspensions of varying MS2 concentrations, as detected by the nested real-time PCR assay

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 (\pm 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 (\pm 1053) RNA copies detected; the recovery level then increased to 0.060%, or 775 (\pm 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 (\pm 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 (\pm 3118) RNA copies; this rate increased again for viruses aerosolised from the suspension of 10^3 PFU/mL, with 1222 (\pm 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.

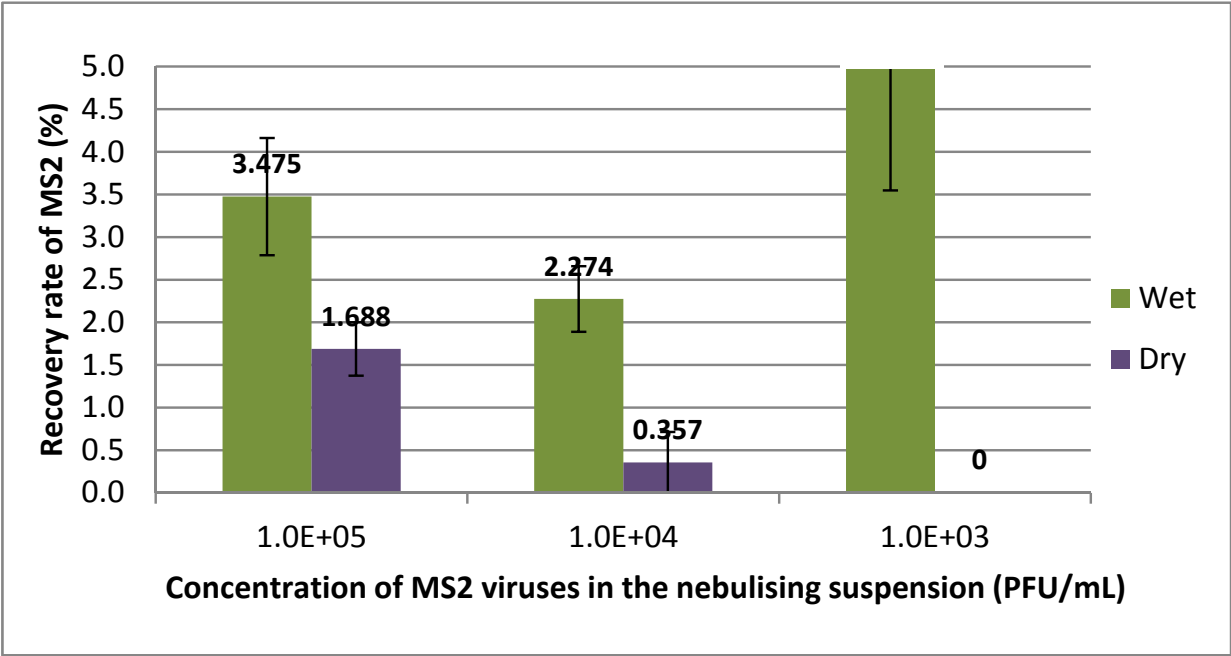


Fig. 8. Recovery of infectious MS2 from aerosols generated from artificial mucous nebulising suspensions of varying virus concentration, as detected by the plaque assay

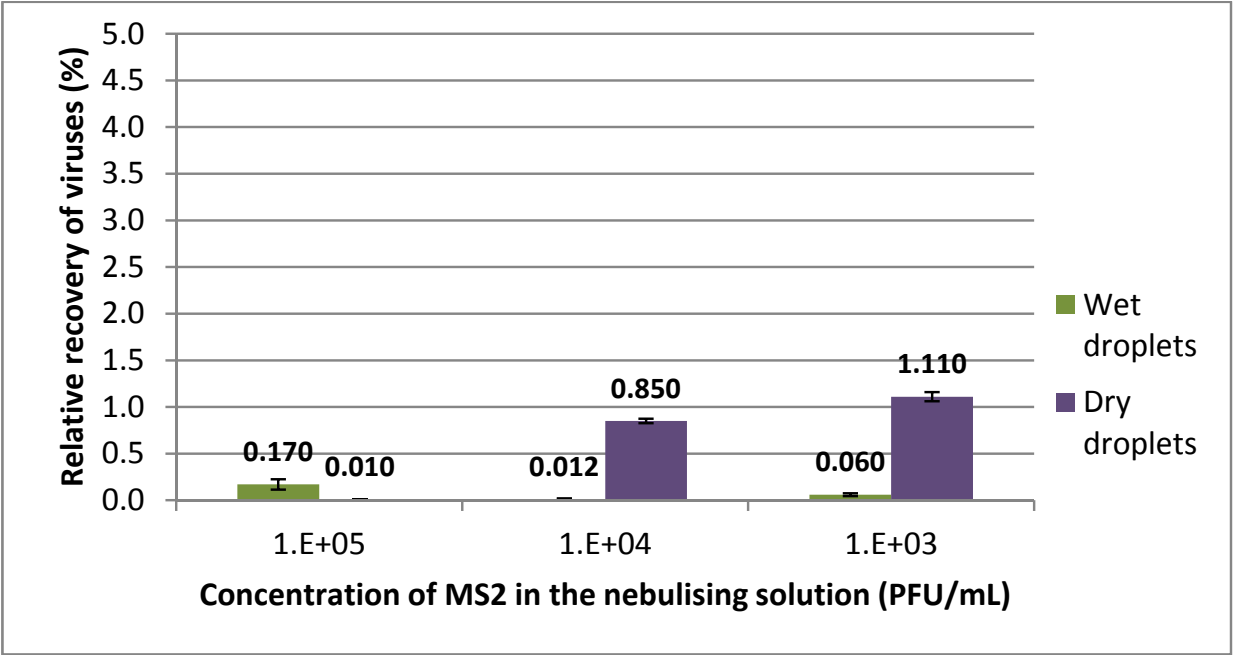


Fig. 9. Recovery of MS2 RNA copies from droplets generated from an artificial mucous suspension of varying MS2 concentration, as detected by the nested real-time PCR assay

3. Conclusions

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 PBS droplets, with relative recovery rates of up to 0.17 % as detected by the plaque assay, and up to 1.03 % as detected by the nested real-time PCR assay. When aerosolised from artificial mucous droplets, MS2 was recovered 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.

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 10⁴ 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 10⁴ PFU/mL for the dry droplets and 10³ PFU/mL for the wet droplets.

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.

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.

4. Significance

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.

In addition to contributing to knowledge in the field of respiratory virus 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 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.

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. 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. 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 study, as well as the method development, can be applied to the study of respiratory viruses in droplets.

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.

We have also conducted aerosol research with influenza virus, which provided surprisingly robust and stable results (data not shown), 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. In any case, it is possible that this may be the case for similar studies with RSV.

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.

5. Acknowledgements

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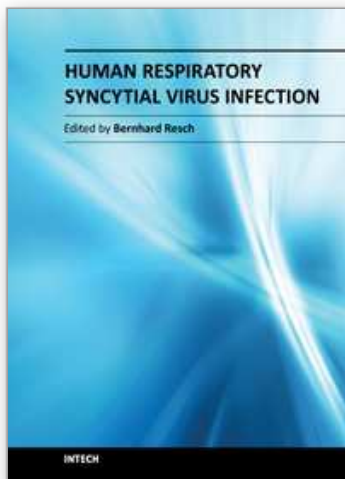
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In this online Open Access book on "Human RSV Infections", several distinguished authors contribute their experience in respiratory syncytial virology. A major focus lies on the fascinating pathophysiology of RSV and represents recent and actual work on different mechanisms involved in the complex pathogenesis of the virus. The second section elucidates epidemiologic and diagnostic aspects of RSV infection covering a more clinical view of RSV disease. At last, treatment modalities including the search for a vaccine that is still not in sight are discussed and conclude this book, thus building up a circle that runs from experimental models of RSV related lung disease over clinical aspects of disease to the latest news of therapeutic and prophylactic approaches to human RSV infection.

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