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

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KEYWORDS

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ABSTRACT

Aim

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.

Method

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 PCR assays were applied in an aerosol study to compare their abilities to detect bacteriophage in air samples.

Conclusions

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.

Significance and Impact

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 survival and fate of respiratory viruses and risks associated with the spread of viruses through aerosol transmission.

BACKGROUND

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, Sandin *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, Ludwig *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, Zhou *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, Safatov *et al*. 2006), and semi-nested or nested PCR (Myatt, Johnston *et al*. 2003). Others have used real-time PCR (Schweigkofler, O'Donnell *et al*. 2004; Zeng, Westermark *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, Pearson et al. 1997; McGoldrick, Lowings et al. 1998; Heath, King et al. 2003; Wu, Xu 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.

MATERIALS AND METHODS

Selection of organisms

In this study, 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 PCR assay was also developed for MS2, a bacteriophage which has been used as a model for airborne viruses including influenza (Foarde, Hanley et al. 1999; Barker and Jones 2005; Hogan, Kettleson 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).

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. 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 1200rpm for 10mins to pellet the bacteria. The supernatant containing the MS2 was filtered with a 0.2μm 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). 0.5mL of each

dilution was added to 3.5mL 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.

Design of nesting primer sets

Nesting 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 as shown. 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.

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 a varied final concentrations (final

concentrations differed for each assay; see table 1) in a 20µL reaction volume. 5µL of PCR product was added to each reaction mix. The reactions were performed on an ABI 7500 system, with the cycling conditions of 50^{o}C for 30 mins (RT step) followed by 95^{o}C for 10 mins, and then 40 cycles of 95^{o}C for 15secs and 60^{o}C for 60secs.

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 (50µL final volume). Primers were added to a final concentration of 0.4pmol/µL. 5µL of RNA sample was added to the mixture per 50µL 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^{o}C for 30mins (RT step); 20 cycles of 94^{o}C for 45secs, 55^{o}C for 45secs, 72^{o}C for 30secs; then 72^{o}C for 7mins. 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^{o}C for 30 mins (RT step) followed by 95^{o}C for 10 mins, and then 40 cycles of 95^{o}C for 15secs and 60^{o}C for 60secs.

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 solution of MS2 (10^6PFU/mL) was prepared in PBS to and each dilution was subsequently aerosolised with a Collison 6-jet nebuliser, operated at 10L/min, inside a chamber. Aerosols were collected onto sterile, empty petri dishes which were previusly 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 560µL 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.

RESULTS

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 2.

The real-time PCR assay for influenza A, subtype H1N1, had an endpoint of 1 $TCID_{50}$, detected at 36.99 Ct. The nested real-time PCR assay, detected the same virus concentration at 20.64Ct. 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 22.17Ct 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 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.

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. The nested real-time PCR assay detected the 10^4 $TCID_{50}$ titre of virus at15.97 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.

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.

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, shown in figure 2, the nested real-time PCR assay amplified MS2 from 14 out of the 16 samples, as seen in figure 3. 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.

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 22.03Ct and 37.11Ct. 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 26.62Ct and 37.73 Ct, with an average of 34.79Ct. 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 22.06Ct, with individual results ranging between 16.57Ct and 37.37Ct. 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 graph 1, 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.

DISCUSSION AND CONCLUSIONS

A series of nested real-time PCR assays for detection of respiratory viruses in aerosols has been successfully developed, 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, Johnston 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.

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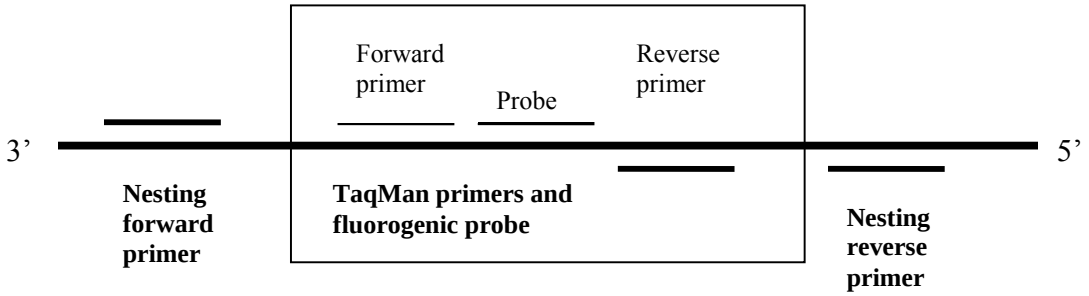


Figure 1. This diagram displays the positioning of the primer sets designed in this study, shown in relation to the existing TaqMan® primers and probe. The new primer sets were designed to nest the TaqMan® target sequences. This was done for each of the viruses in this study.

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	TGACATCAACGACAACAGGAAATCATGTTCTG	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

Table 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.

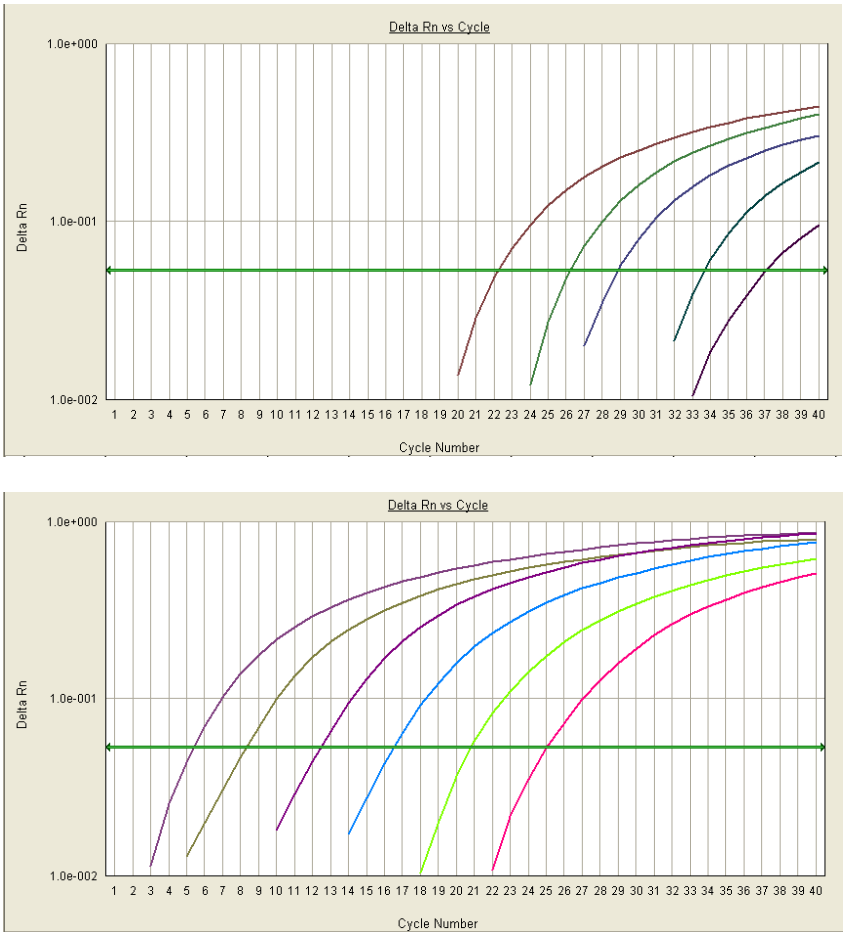


Figure 2. This figure shows the comparison of the real-time PCR assay (top) with the nested real-time PCR assay (bottom) for a 10-fold dilution series of influenza A, subtype H1N1. The concentrations of H1N1 virus tested ranged from 10^4 $TCID_{50}$ down to 0.1 $TCID_{50}$. The nested real-time PCR assay 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.

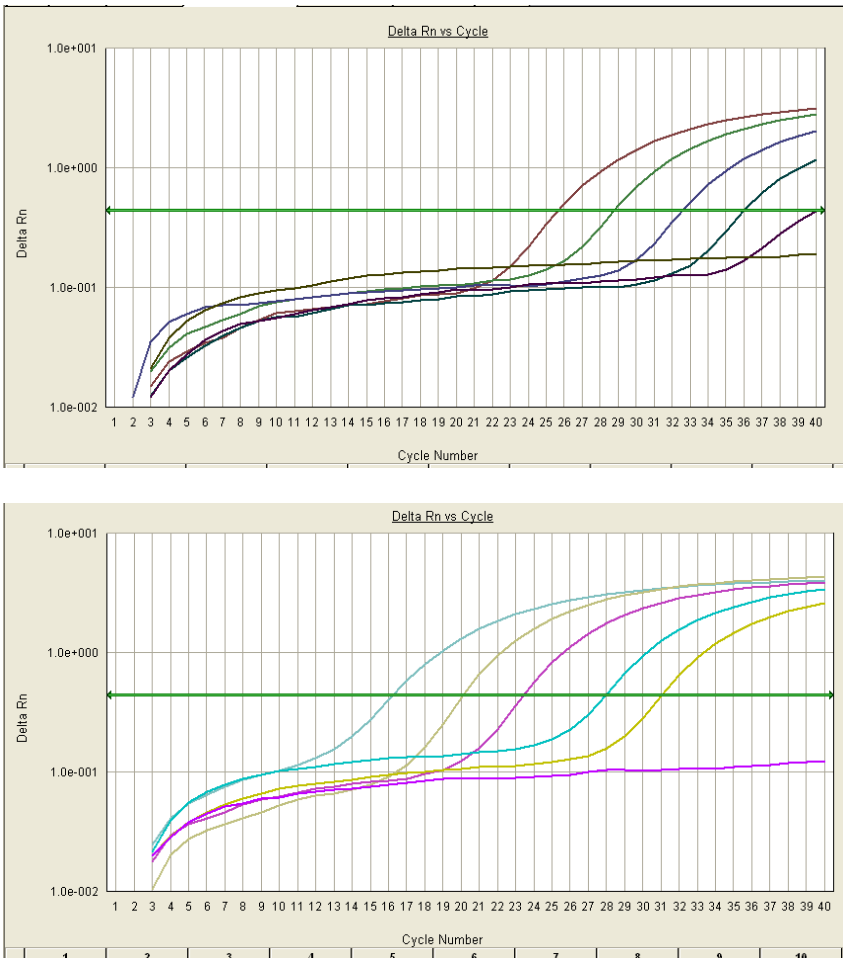
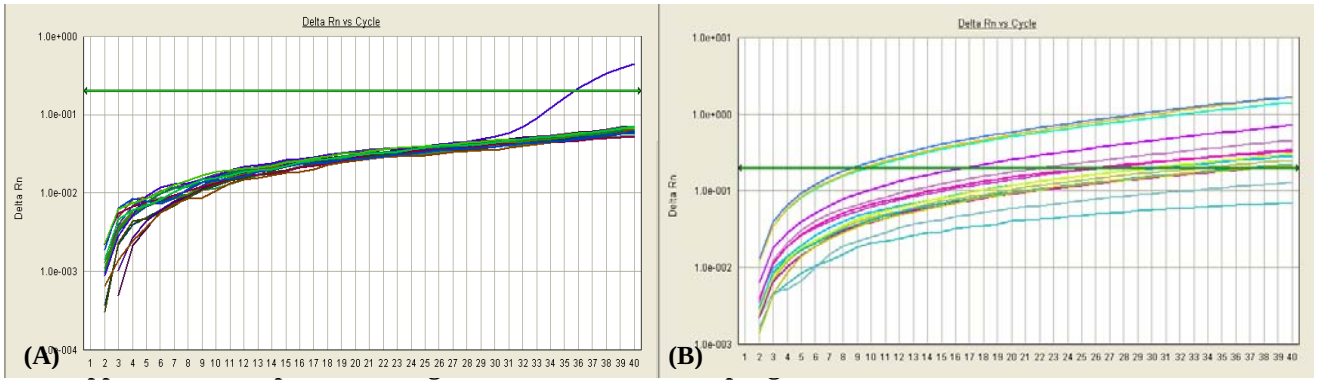


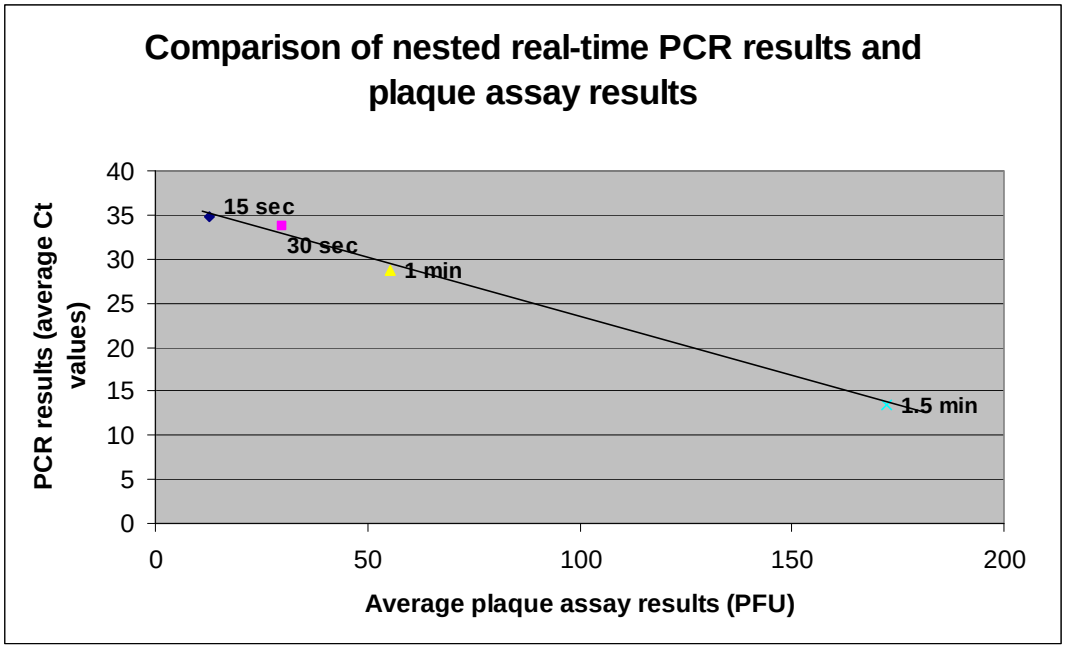
Figure 3 This figure shows the comparison of the real-time PCR assay (top) with the nested real-time PCR assay (bottom) for a 10-fold dilution series of human respiratory syncytial virus (RSV). The highest concentration of RSV tested was from 10^4 $TCID_{50}$ down to 0.1 $TCID_{50}$. The nested real-time 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.

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

Table 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 titre (PFU)		10^4	10^3	10^2	10	1	0.1
MS2	Real-time	23.11	26.33	30.63	33.50	ND	ND
	Nested	7.42	9.27	12.39	16.02	19.18	19.31





Graph 1 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).