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INVESTIGATIONS INTO HUMAN INFLUENZA TRANSMISSION

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for the degree of Doctor of Philosophy**

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Summary of the work in this thesis

Limited understanding of influenza transmission has been a frequent obstacle during the development of pandemic influenza infection prevention and mitigation strategies. The science is hotly debated, especially the relative importance of transmission via large droplets or aerosols. Clarification of the relative importance of different modes of transmission is critical for the refinement of evidence-based infection control advice and has been called for by the European Center for Disease Control (ECDC), the World Health Organization (WHO), and the US Institute of Medicine.

The primary aims of this thesis were to investigate influenza transmission; i) by obtaining data concerning viral shedding and the presence of influenza virus in the near environment of infected individuals and ii) through the exploration of a human challenge model to study transmission.

Two major clinical studies have been performed;

- Shedding and environmental deposition of novel A (H1N1) pandemic influenza virus. The primary aims of the study were to correlate the amount of virus detected in a subject's nose with that recovered from his/her immediate environment (on surfaces and in the air) and with symptom duration and severity. Adults and children, both in hospital and from the community, who had symptoms of influenza infection were enrolled. Information about symptoms was collected and samples were taken including nose swabs, swabs from surfaces and air samples. Forty two subjects infected with influenza A(H1N1)pdm09

were recruited and followed up. The mean duration of nasal viral shedding was 6.2 days (by PCR) and 4.6 days (by culture). Over 25% of cases remained potentially infectious for at least 5 days. Symptom scores and viral shedding were poorly correlated. From surface swabs collected in the vicinity of 40 subjects, 15 (38%) subject locations were contaminated with virus. Overall 36 of 662 (5.4%) surface swabs taken were positive for influenza, two (0.3%) yielded viable virus. Subjects yielding positive surface samples had significantly higher nasal viral loads on illness Day 3 and more prominent respiratory symptom scores. Room air was sampled in the vicinity of 12 subjects and PCR positive samples were obtained from five (42%). Particles small enough to reach the distal lung ($\leq 4\mu\text{m}$) were found to contain virus.

- Use of a human influenza challenge model to assess person-to-person transmission: Proof-of-concept study. The primary aim of this study was to establish that an experimentally induced influenza infection is transmissible. Healthy subjects deemed sero-susceptible to influenza A/H3N2/Wisconsin/67/2005 were intranasally inoculated (Donors) and when symptoms began, further sero-susceptible subjects (Recipients) were exposed to Donors during an 'Exposure Event'. Subjects were in close contact, e.g. playing games and eating meals together, for a total of 28 hours during a 2 day period. Samples were collected to confirm infection status. Among 24 healthy adult subjects, nine were randomised to the 'Donor' group and 15 to the 'Recipient' group. Following inoculation 5 out of 9 Donors (55%) developed illness and 7 out of 9 (78%) were proven to be infected. After exposure, 5 out of 15 Recipients developed symptoms and 3 out of 15 were proven to be infected. Three others were found to be non sero-susceptible prior to

exposure. The overall attack rate in Recipients was 20% but was 25% after adjustment for pre-exposure immunity.

The contact, droplet and aerosol routes of influenza transmission are all likely to have a role. This thesis shows that transmission of influenza via surfaces may be less important than current infection control policies and public guidance documents imply. Air sampling results add to the accumulating evidence that supports the potential for aerosol transmission of influenza. The human challenge model could be used to investigate routes of influenza transmission further and a study funded by the Centers for Disease Control (CDC) is planned.

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS WORK

Original Research

Killingley B, Enstone JE, Greatorex J, Gilbert AS, Lambkin-Williams R, Cauchemez S, Katz JM, Booy R, Hayward A, Oxford J, Bridges CB, Ferguson NM, Nguyen Van-Tam JS. Use of a human influenza challenge model to assess person-to-person transmission: Proof-of-concept study. *J Infect Dis.* 2012 Jan; 205(1):35-43.

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Reviews / Editorials

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Killingley B. Respirators versus medical masks: Evidence accumulates but the jury remains out. *Influenza Other Resp Viruses.* 2011 May;5(3):143-5.

Killingley B and Nguyen Van-Tam J. A scientific review of the proposed routes of influenza transmission and their relative significance.

Department of Health, England, available at;

http://www.dh.gov.uk/prod_consum_dh/groups/dh_digitalassets/documents/digitalasset/dh_125332.pdf.

Book Contributions

Killingley B & Enstone JE. Influenza transmission and related infection control issues. Chapter 8, Introduction to Pandemic Influenza; Second Edition. Edited by Jonathan Van-Tam & Chloe Sellwood; CABI Publishing, In Press.

Presentations

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What can outbreak reports tell us about influenza transmission? Oral presentation: Workshop on influenza transmission, CDC Atlanta, US; Nov 2010.

Influenza challenge model. Oral presentation: Options for the control of influenza conference, Hong Kong; Sept 2010.

Influenza virus shedding and environmental deposition. Poster presentation: Options for the control of influenza conference, Hong Kong; Sept 2010.

Influenza transmission – A challenging subject. Oral presentation: Federation of Infection Services conference, Birmingham; Nov 2009.

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Abbreviations

A(H1N1)pdm09	The pandemic influenza A(H1N1)2009 virus
AE	Adverse Event
AH	Absolute Humidity
AR	Attack Rate
ARI	Acute Respiratory Infection
BP	Blood Pressure
CCU	Common Cold Unit
CDC	Centers for Disease Control
CI	Confidence Interval
COPD	Chronic Obstructive Pulmonary Disease
Ct	Cycle threshold
DH	Department of Health
ECDC	European Centre for Disease Control
ECG	Electrocardiogram
ELISA	Enzyme Linked Immunosorbent Assay
FBC	Full Blood Count
FFP	Filtering Face Piece
GCP	Good Clinical Practice
GM	Geometric Mean
GMP	Good Manufacturing Practice
GP	General Practitioner
HA	Haemagglutinin
HAI	Haemagglutination Inhibition
HCW	Healthcare Worker
HH	Hand Hygiene
HID	Human Infectious Dose

HIV	Human Immunodeficiency Syndrome
HPA	Health Protection Agency
ICH	International Conference on Harmonization
ID ₅₀	Infectious Dose (causing a 50% infection rate)
IF	Immunofluorescence
ILI	Influenza-Like Illness
LRT	Lower Respiratory Tract
MD	Mean Difference
MDCK	Madin-Darby Canine Kidney
MN	Microneutralisation
MRC	Medical Research Council
NA	Neuraminidase
NAI	Neuraminidase Inhibitor
NHS	National Health Service
NIHR	National Institute for Health Research
NIOSH	National Institute of Occupational Safety and Health
NPA	Nasopharyngeal Aspirate
NPI	Non Pharmaceutical Interventions
OR	Odds Ratio
PA	Plaque Assay
PB1	Polymerase Basic protein 1
PB2	Polymerase Basic protein 2
PCR	Polymerase Chain Reaction
PCT	Primary Care Trust
PFU	Plaque Forming Units
PPE	Personal Protective Equipment
R0	Reproductive Number
RCT	Randomised Control Trial

REC	Regional Ethics Committee
RH	Relative Humidity
RNA	Ribonucleic Acid
RR	Respiratory Rate
RSV	Respiratory Syncytial Virus
rt-PCR	Reverse Transcription Polymerase Chain Reaction
RVL	Retroscreen Virology Limited
SAE	Serious Adverse Event
SAR	Secondary Attack Rate
SARS	Severe Acute Respiratory Syndrome
SFM	Surgical Face Mask
SI	Serial Interval
SOP	Standard Operating Procedure
SRAT	Short Range Aerosol Transmission
SS	Systemic symptoms
TCID ₅₀	Tissue Culture Infectious Dose (causing a 50% infection rate)
TS	Total Symptom
UK	United Kingdom
UKCRC	United Kingdom Clinical Research Collaboration
UoN	University of Nottingham
URT	Upper Respiratory Tract
URTI	Upper Respiratory Tract Infection
US	United States
UV	Ultra Violet
VTM	Viral Transport Medium
WHO	World Health Organization

Chapter 1:

Introduction

1.1 Global Health Threat

Influenza is recognised as a significant threat to human health across the world. This threat comes from annual epidemics (seasonal influenza) and less frequent pandemics. It is estimated that seasonal influenza causes 4 million episodes of severe illness and between 250,000 and 500,000 deaths every year (WHO 2011a). In industrialised countries, it is the elderly and those with co-morbidities who bear the brunt of disease mortality and morbidity (Thompson *et al*, 2004; Mullooly *et al*, 2007). As well as causing illness it also exacts an economic burden through healthcare costs and lost productivity. In the United States (US) in 2003, the total costs associated with influenza were estimated to be \$87 billion (Molinari *et al*, 2007).

It is pandemic influenza however, that has the potential to cause a major, global health emergency causing severe illness and significant societal disruption. In order to have pandemic potential a virus must satisfy the following criteria; i) be of a novel sub-type, i.e. the haemagglutinin is unrelated to immediate (pre-pandemic) predecessors; ii) little or no pre-existing population immunity should exist; iii) causes significant clinical illness; and iv) exhibit efficient person to person spread (WHO 2009a).

These factors will allow a virus to infect many and spread rapidly.

Estimating deaths from pandemic influenza is difficult. Data from the US suggest that mortality during the 2009 pandemic was comparable to a severe seasonal epidemic, though the years of life lost may have been higher due to relative impact on young adults. Viboud *et al* estimate that between 7,500 and 44,100 deaths were attributable to the A(H1N1)pdm09 virus in the US during May-December 2009, and that between 334,000 and 1,973,000 years of life were lost. This range of years of life lost

includes at its lower bound the impact of a typical influenza epidemic dominated by the more virulent H3N2 subtype, and at its upper bound the impact of the 1968 pandemic (Viboud *et al*, 2010). Extrapolating these figures to the rest of the world indicates that approximately 500,000 people may have died. This compares with estimates of at least 50 million deaths in the 1918 pandemic (Potter 2001). The reasons for the huge mortality rates seen in 1918/1919 are not completely understood but may include factors such as viral virulence, secondary bacterial infections, poor healthcare and lack of antibiotics and the occurrence of the pandemic during wartime (which saw mass movements of people and poor standards of living).

Despite advances in our knowledge about influenza and the ability to treat infection and its complications, the emergence in 1997, of avian influenza infections in man associated with mortality rates of over 50%, have led to fears that a pandemic on the scale of 1918 could occur at any time. The impact that such a disease would have on the connected and globalised world we see today could be enormous. As a result preparations to plan for and mitigate the threat posed by an avian related influenza pandemic have taken place and are on-going. Planning efforts have been spearheaded by WHO which has defined six phases of preparedness. Key actions during the inter-pandemic periods (phases 1 and 2) include producing, implementing and exercising national preparedness and response plans, whilst features of the response during a pandemic (phase 6) include containment of the emerging virus and strategies to mitigate the effects on society (WHO 2009b).

1.2 Virology

Influenza belongs to the Orthomyxoviridae family of ribonucleic acid (RNA) viruses. Three types of influenza exist; A, B and C which are classified serologically based on major antigenic differences. Influenza A has a diverse array of animal hosts including birds, humans, horses, pigs and marine mammals. Influenza B and C on the other hand are essentially restricted to humans. Influenza A causes the most common and the most serious infections and has the potential for both epidemic and pandemic spread. In contrast influenza B and C tend to cause milder disease and do not cause pandemics (Zambon 2001). The discussion that follows is largely limited to influenza A.

Influenza A is an enveloped virus that possesses negative sense RNA which is organised into eight gene segments that encode nine structural and one or two non-structural proteins. The virus exists in many different subtypes, based on antigenic differences in its envelope glycoproteins which either possess haemagglutinin (HA) or neuraminidase (NA) activity. Sixteen HA and nine NA subtypes have been identified to date (Obenauer *et al*, 2006). These glycoproteins have a critical role to play in binding host cell receptors. The HA proteins project from the envelope surface creating a host cell receptor binding site, whilst the NA protein has enzymatic activity which removes terminal sialic acids from glycoproteins and plays a role in the release of progeny virus from infected cells. Only HA types 1, 2 and 3 and NA types 1 and 2 are currently responsible for stable (as opposed to sporadic) human infections.

Avian influenza viruses in aquatic birds serve as the main reservoir for all known subtypes of influenza A. Interspecies transmission of influenza can

occur but it is restricted by certain host factors. The determinants of host specificity are not entirely clear but almost certainly involve the HA and NA glycoproteins as well as the polymerase basic protein 2 (PB2). The role of HA has been shown by studying the receptors on epithelial cells. In man influenza viruses have a preference for receptors showing sialic acid – galactose linkages of $\alpha 2,6$, where as avian viruses in comparison show preference for $\alpha 2,3$ linkages (Zambon 2001). Interestingly the pig shows $\alpha 2,6$ and $\alpha 2,3$ type receptors in respiratory epithelial cells which explains why this animal is susceptible to both human and avian viruses (Ito *et al*, 1998).

Human adapted strains are thought to arise through two separate mechanisms; i) direct transmission from birds with subsequent virus adaptation in a human host, ii) transmission from birds via an intermediate host e.g. a pig. In this instance, the pig serves as a 'mixing vessel' for avian and human viruses where viral RNA can be exchanged resulting in a reassorted virus capable of causing human disease (Scholtissek *et al*, 1985; Kida *et al*, 1994).

The evolutionary success of influenza can be ascribed to two main factors (Webster *et al*, 1997);

1. The animal reservoir in birds and the large number of possible strains
2. The ability of the virus to undergo genetic change

Two processes allow for genetic variation of the virus; antigenic drift and antigenic shift. The changes that result can allow the virus to evade humoral immunity and this provides a selection advantage. Antigenic drift is brought about by the infidelity of the RNA polymerase. This leads to amino acid changes in the major antigenic proteins HA and NA (other

proteins are more conserved) with the result that every 1-2 years, the predominant strain of influenza A is replaced by a variant able to evade existing antibody responses. Antigenic shift is the result of viral reassortment, a process facilitated by the segmented nature of the viral genome. Reassortment occurs when two different viruses meet in the same cell and exchange genetic material. The 1957/58 pandemic for example, was caused by a reassortment event between a human H1N1 virus and an avian H2N2 virus. The HA, NA and polymerase basic 1 (PB1) genes of the avian virus combined with the five remaining human origin genes to create an H2N2 reassortant virus capable of causing disease and being transmitted by humans (Scholtissek *et al*, 1978). Such viruses may not have the ability to infect human cells initially but given evolutionary time, adaptations may occur and indeed the 1918 pandemic virus may have originated in this way (Taubenberger *et al*, 1997).

1.3 Pathology

In humans influenza viruses replicate in epithelial cells lining both the upper and lower respiratory tree. Non fatal cases tend to involve the upper tract whilst most fatal cases are associated with pneumonia (Taubenberger and Morens 2008). The HA molecules bind to cell surface receptors and once the virus has gained entry to a cell, replication occurs followed by release of progeny virus. This process ultimately leads to cell death by a number of mechanisms including loss of critical cellular proteins and apoptosis.

It is likely that cell death and associated inflammation are the cause of the typical respiratory tract symptoms whilst systemic effects such as fever

and myalgia are caused by inflammatory mediators such as cytokines. Concomitant bacterial infection can often complicate the picture and organisms such as *Staphylococcus aureus*, *Haemophilus influenzae* and *Streptococcus pneumoniae* are frequently implicated. The well recognized propensity to bacterial super-infection may be related to impairment of ciliary clearance mechanisms, increased bacterial adherence to epithelial linings and defects in neutrophil and mononuclear cells caused by virus infection (Treanor 2010). The host response to infection involves both innate and adaptive responses. Adaptive responses include the production of strain-specific antibodies, which exert selective pressure on circulating influenza viruses and which drive antigenic drift of seasonal influenza viruses, especially in the hemagglutinin molecule. This antigenic drift necessitates updating of seasonal influenza vaccines regularly in order to match the circulating strains. Infection also induces virus-specific T cell responses which are mainly directed to conserved proteins. Both T cell and to a lesser extent antibody mediated immunity contribute to so-called heterosubtypic immunity; immune responses which show cross-reactivity with a variety of influenza A viruses of different subtypes and may afford protection against antigenically distinct, influenza viruses (Kreitz *et al*, 2011).

1.4 Epidemiology

In general three distinct epidemiological forms of influenza disease are recognized; local outbreaks, epidemics and pandemics.

1.4.1 Epidemics

An influenza epidemic is defined as an outbreak of the disease, with numbers of cases above that normally expected in a given population over a given period of time. The HPA has defined the baseline threshold for 'normal seasonal activity' in England as 30 GP consultations per week/100,000 population. The epidemic threshold would be reached if the number of consultations surpassed 200 per week/100,000 (HPA 2012). They are usually associated with minor antigenic changes (drift) of previously circulating viruses. The number of cases rises sharply over 2-3 weeks and peaks at around week 6. Epidemics tends to finish rather abruptly, the reasons for this are not entirely clear and cannot be attributed solely to a lack of susceptible persons. The typical attack rate during an epidemic is 10-20% of susceptible persons though this can be higher in specific age groups e.g. schoolchildren. Clinically an epidemic is characterized by an increase in febrile respiratory illness in children, followed by influenza like illness (ILI) in adults which triggers an increase in hospital admissions [typically exacerbations of Chronic Obstructive Pulmonary Disease (COPD) and pneumonia] and work absenteeism (Monto and Kioumehr 1975; Glezen *et al*, 1987).

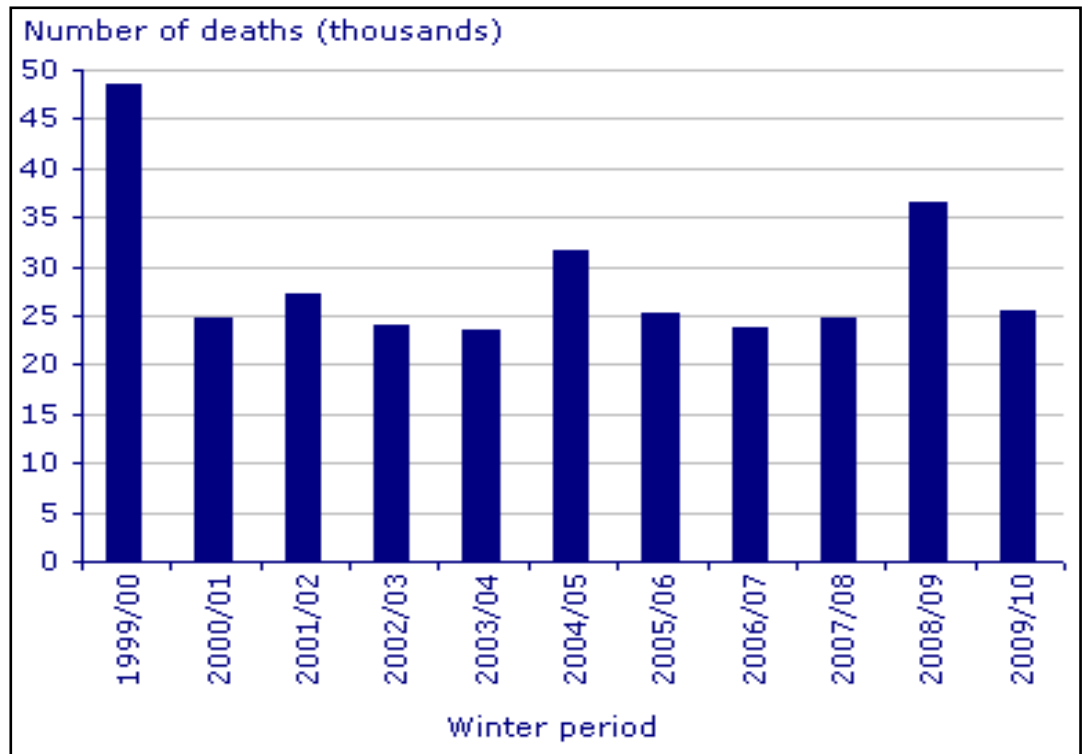
Until 2009, strains of influenza A H1N1 and H3N2 had been co-circulating worldwide. In the years immediately prior to 2009 influenza activity in the United Kingdom (UK), as measured by General Practitioner (GP) consultation rates had been low (Fleming and Elliot 2008). In fact the last

time the UK experienced an epidemic was in 2000 (Figure 1.1). This reflects the degree of population immunity that built up due to continuing presence of both subtypes and a lack of significant antigenic drift. It remains to be seen what the effect of the 2009 pandemic and the resultant dominance of the H1N1 subtype will have on outbreaks in the coming years.

Excess deaths are well known to occur during influenza seasons but estimating the true burden that influenza exacts on populations is not straightforward and a variety of measures such as GP consultations, hospitalisations and deaths are used to illustrate it. The data generated however is often insensitive, being complicated by the often non specific nature of influenza illness, lack of microbiological diagnosis and secondary infections/complications which can lead to inaccurate hospital episode records and death certification. Data from between 1996 and 2004 have been used to provide estimates of disease burden in the UK (Pitman *et al*, 2007). Between 10,500 – 25,000 deaths, 800,000 GP consultations and 28,000 hospital admissions were attributable to influenza (A+B) annually. The elderly account for the majority of deaths whilst the young, particularly pre-school children, suffer the highest attack rates. Hospitalisations and death rates are very much more increased in those who have underlying 'high risk' conditions which complicate influenza infection e.g. chronic cardiac, pulmonary and renal conditions, diabetes and immunosuppressed states (Barker and Mullooly 1980). In ageing populations such as those seen in many developed countries, such conditions are more prevalent and this has been associated with increasing hospitalization and death rates over recent decades (Thompson *et al*, 2003). The predominance of the H3N2 subtype over the last two decades

has played a significant role as this has been shown to be a more virulent virus than H1N1 (Kaji *et al*, 2003; Thompson *et al*, 2003).

Figure 1.1: Excess winter deaths, England and Wales, 1999/2000–2009/2010. Source; (Office for National Statistics 2010).



1.4.2 Pandemics

Influenza pandemics have affected man throughout history; it has been speculated that since 1500 there have been at least 14 (Potter 2001; Taubenberger and Morens 2009). Two conditions must be met for an outbreak of influenza to be called a pandemic. The first is that the virus must be novel (specifically the HA) and the second is that the virus having originated in a specific location must spread around the world. Spread is facilitated by the presence of susceptible populations that have no significant pre-existing immunity. Clinical attack rates in the range of 25-

40% are seen. The pandemic of 1918-19 is thought to have affected 25% of the world's population and caused the death of at least 50 million people (Johnson and Mueller 2002) whilst the pandemics of 1957-58 and 1968-69 are thought to have been responsible for the deaths of 1 and 0.5 million people respectively (Potter 2001). The occurrence and severity of pandemics can be difficult to predict as exemplified by the 2009 pandemic. Preparedness activities prior to 2009 had focused on a pandemic virus originating in Asia (because of the high density and close proximity of pig, bird and human populations) and had assumed that high attack and mortality rates would be encountered. Instead, disease emerged in Mexico (the origins of the virus itself remain uncertain) and it was ultimately observed to be a mild infection in the vast majority (Writing Committee of the WHO Consultation on Clinical Aspects of Pandemic Influenza 2010).

1.4.3 Epidemiological uncertainties

Some epidemiological aspects of influenza remain uncertain; for example, where does the virus reside during periods of relative inactivity? What forces determine the appearance of epidemics? Why are epidemics so explosive?

For some infectious diseases (e.g. measles) outbreaks can be predicted.

Infections appear when levels of population immunity fall below certain thresholds, i.e. a certain proportion of susceptible individuals exist.

Influenza however, does not necessarily show such simple dynamics. The fact that the virus is not antigenically stable and the influence of host and environmental factors make the epidemiology of influenza more complicated. A case in point is the seasonality of influenza.

Both influenza outbreaks and epidemics show marked seasonality. In temperate parts of the world viruses are thought to persist at low levels throughout the year but exhibit a marked increase most often in winter. Influenza also occurs in tropical climates; however, here infections tend to occur more evenly throughout the year. The reasons for this seasonality in temperate zones remain to be conclusively proven and many theories abound (Lofgren *et al*, 2007; Cannell *et al*, 2008; Lowen *et al*, 2008; Tamerius *et al*, 2010).

- Environmental conditions – transmission of influenza is affected by temperature and humidity (Lowen *et al*, 2007) with absolute humidity being most strongly associated (Shaman and Kohn 2009); low temperature and low humidity are known to favour virus survival (McDevitt *et al*, 2010 & Hood 1963). Reduced levels of UV light in winter time may facilitate airborne virus survival (but only outdoors) (Sagripanti and Lytle 2007). Prevailing air currents may carry airborne virus across the globe at certain times of the year (Hammond *et al*, 1989).
- Host susceptibility – it has been suggested that host resistance to infection wanes in winter; the effect of temperature and humidity on airways (Shadrin *et al*, 1977) and levels of vitamin D (Cannel *et al*, 2008) and melatonin (Dowell 2001) have been implicated. Viral interference (the inhibition of influenza infection caused by infection with other respiratory viruses) has also been proposed as a factor (Linde *et al*, 2009).
- Contact rates – people tend to spend more time indoors during winter time or rainy seasons and schools (which are regarded as important transmission sites) are closed for prolonged periods during summer (Lofgren *et al*, 2007).

It is unlikely that one theory alone can explain the phenomenon and it may well be a complex interaction of mechanisms that is responsible.

1.5 Influenza Transmission and Infection Control

A key part of protecting the public from the threat of influenza is being able to prevent or reduce the spread of infection. Mitigation strategies can be population orientated (e.g. vaccination and social distancing) or more individualised (e.g. hand hygiene and the use of face masks) but the success of each is largely influenced by the nature of infection transmission. Furthermore, during a pandemic many non-pharmaceutical interventions may assume greater significance in limiting transmission than pharmaceutical interventions (e.g. vaccines and antivirals) which may not be available to all.

1.5.1 Transmission

Influenza is a disease of the respiratory tract and this is reflected in the symptoms caused by infection, e.g. cough, sore throat, blocked nose.

Infection spreads when virus from the respiratory tract of one person is transmitted to the respiratory tract of another person. Infection transmission can occur by three routes, all of which begin with the production of virus containing particles by actions such as coughing and sneezing. It is the size and fate of these particles that determines which route(s) of infection may act;

- Large droplets; these particles can deposit on mucous surfaces of the upper respiratory tract (URT) such as the mouth and nose. They can be inspired but not respired (they are too large to reach the lungs).

- Droplet nuclei (hereafter referred to as aerosols); these particles are small enough to be respired and reach the lower respiratory tract (LRT) and they may also deposit on surfaces in the URT.
- Contact transmission; Particles are collected on the hand(s) and then transferred to mucous membranes of the upper respiratory tract. This can occur as a result of direct contact with an infected person or via contact with particles which have been deposited in the environment e.g. on surfaces.

1.5.2 Infection Control

Two broad categories of infection control precautions can be considered in healthcare settings. Standard infection control precautions should be followed during every encounter between a healthcare professional and a patient on the basis that many transmissible infections may not be immediately evident. Standard precautions include environmental hygiene, hand hygiene and the use of personal protective equipment (gloves and aprons). Transmission based infection control precautions build on standard precautions and include measures to protect against specific types of transmission when a particular infection is suspected or has been confirmed. These specific precautions cover droplet, contact and 'airborne' (referring to aerosol) transmission.

Although the relative importance of the routes of influenza transmission are hotly debated, practically (and historically) influenza is regarded as being transmitted primarily by the droplet and/or contact route of transmission and infection control guidance from different countries reflects this (Department of Health 2009; CDC 2010b). On this basis the following standard and droplet based infection control precautions are recommended to prevent or reduce the transmission of influenza (NB

whilst these precautions are aimed primarily at healthcare settings, many of them are also applicable to the wider community):

- Distancing; close contact between people has been consistently demonstrated in epidemiological studies to be associated with influenza transmission and this observation has led to a number of recommendations.
 - Those with acute symptoms of influenza are advised to isolate themselves from others. This might involve taking time off work or school and staying at home. In a healthcare setting this would entail placing patients in isolation rooms or cohorting a group of patients in a specific area.
 - Based on knowledge of how far droplets can spread a 'safety distance' of between 1-2m around infected patients is used; within this zone facemasks should be worn.
- Environmental hygiene; settled droplets pose a risk for contact transmission and simple cleaning of commonly touched surfaces with water and detergents or other household cleaning agents can effectively remove virus.
- Respiratory hygiene includes the use of hands and tissues to catch respiratory droplets that are released during coughing and sneezing. Tissues should be thrown away and hands washed after each event.
- Hand hygiene has been shown to be effective in reducing a variety of respiratory and gastrointestinal infections. By removing virus from hands, the contact route of transmission is interrupted.
- Personal protective equipment includes the use of disposable gloves and aprons to prevent contamination of the wearer's hands and clothes respectively.
- Surgical face masks (SFMs) were initially used by healthcare workers (HCWs) undertaking surgical procedures to help maintain a sterile field

but their use to protect HCWs from infectious patients is also longstanding, dating back to the 1918 pandemic. SFMs act as a barrier to droplets and can be used in two ways to prevent/reduce influenza transmission. They can be worn by an infected person to block the release of droplets or they can be worn by a susceptible person to prevent droplets reaching their URT. By virtue of the fact that they cover the nose and mouth they can also reduce hand to face contact and therefore contact transmission. The use of masks is not generally recommended for the public for a number of reasons including the fact that if not used or disposed of properly they could pose an infection risk themselves.

In some circumstances the use of aerosol (airborne) precautions in addition to standard and droplet precautions should be put in place, for example when known aerosol generating procedures are taking place (e.g. tracheal intubation, bronchoscopy). These precautions might include the use of ventilation systems, ultraviolet light and respirators. Respirators are face masks that are designed to protect the wearer from aerosols. They are able to filter out the small particles (droplet nuclei) that constitute an aerosol whereas SFMs in general do not. Respirators however, do have some drawbacks; they are relatively expensive compared to SFMs, they require the wearer to undergo a fit test to ensure that a mask fits and functions properly and they can be uncomfortable to wear for prolonged periods.

Influenza poses some specific challenges to infection control. Firstly, the pressures brought to bear on healthcare systems during a pandemic may be enormous, magnifying the need to prevent nosocomial infection and to reassure and protect staff. Secondly, there may be instances during a

pandemic when the general public are advised to practice transmission based precautions (e.g. use of SFMs) and finally pharmaceutical control of disease (through vaccination or antivirals) may be deficient because of poor uptake, unavailability or sub-optimal response. The importance therefore of evidence based non-pharmaceutical infection control recommendations, especially in the setting of a disease causing high morbidity and mortality cannot be overstated. However, a critical question for influenza infection control is what is the relative significance of each of the routes of transmission? The answer to this question is unknown and in particular the role played by aerosols is unclear. This has led to considerable debate about the need to protect HCWs from aerosol transmission of influenza and which face masks to use. It is not surprising that the European Centre for Disease Control (ECDC), the US Institute of Medicine (IOM), and the WHO have all prioritized understanding the modes of influenza transmission as an important requirement for pandemic planning (ECDC 2007; IOM 2007; WHO 2009c).

1.6 Exploring Transmission Research Gaps

To provide the best infection control advice we need to know the precise details about how influenza is transmitted and the factors that may affect this. Nearly a century has passed since the first studies of influenza transmission were conducted and many questions remain unanswered, for example;

- What is the relative significance of the different routes of influenza transmission?
- Do transmission routes differ in different settings?
- What is the extent and significance of virus deposition in the environment?
- What environmental factors influence transmission?
- What is the relative effectiveness of hand hygiene, SFMs and respirators in preventing transmission?
- What other interventions may be used to reduce transmission?
- How important is transmission from asymptomatic and pre-symptomatic individuals?

The research contained within this thesis has attempted to explore some of these questions, including consideration of how best to answer them. In the next section, the current evidence base concerning influenza transmission is examined with a particular focus on influenza epidemiology, determinants of transmission and routes of transmission. This literature review provides the platform on which further study can be built. Research undertaken that contributes to this thesis, along with discussion is presented in Chapters 3-5, before a final section draws conclusions and looks to the future.

Chapter 2:

Literature Review

Reducing the impact and spread of influenza epidemics and pandemics is central to national and international preparedness plans. Mitigation strategies can include vaccination, antiviral use and social distancing, but their effectiveness is highly dependent on a precise and detailed knowledge of both the epidemiology (dynamics) and biology (determinants) of influenza transmission. Similarly the effectiveness of more individualised infection control recommendations such as hand hygiene and the use of face masks depend on how infection is transmitted. What follows is a review of the literature and evidence base that underpins our current understanding of influenza transmission and it is from this platform that the investigations conducted as part of this thesis were developed. It begins with an overview of influenza epidemiology and explores other factors affecting person-to-person transmission before going on to consider routes of transmission in more detail.

2.1 Influenza Epidemiology

A range of specific measurements have been defined in order to assess the transmissibility of an infectious disease. For influenza most of these measurements have been calculated in relation to household transmission; the home is regarded as the place where the frequency and intensity of infectious contact is highest. These measurements offer insights into the complex nature of disease transmission and have a crucial role to play in our attempts to control disease.

2.1.1 Reproductive number

The reproductive number (R_0) is defined as the average number of secondary cases which one typical infected case would produce in a completely susceptible population. It gives a measure of how a disease

might spread through a population; an R_0 of >1 means that a disease will spread whilst an $R_0 < 1$ means that a disease will die out. R_0 depends on the characteristics of the infectious agent (e.g. infectivity and duration of infectiousness) and of the population (e.g. population density and social mixing patterns). It can therefore differ between infections in the same population but also for the same infection in different populations. Measles is regarded as a highly transmissible disease. In England and Wales the R_0 has been estimated to be between 14-18, i.e. on average one infected case will pass on the infection to 14-18 others (Anderson and May 1982). The coronavirus that caused the severe acute respiratory syndrome (SARS) epidemics in 2003 was estimated to have a R_0 of between 2-5 over a range of countries that were affected (Wallinga and Teunis 2004). Influenza by comparison often has a R_0 of <2 but because of genetic variation of the influenza virus brought about by antigenic drift and shift this can vary both between and within epidemics/pandemics. Based on historical estimates and empiric data the R_0 of influenza can be classified as high ≈ 2 , moderate ≈ 1.7 and low ≈ 1.4 ; pandemic viruses tend to have higher values whilst viruses that circulate during inter-pandemic periods have lower ones (Ferguson *et al*, 2006). A review of studies that reported the R_0 for the 2009 pandemic virus showed a range of 1.2–2.3 with a median of 1.5 (Boelle *et al*, 2011).

2.1.2 Serial Interval

The serial interval (SI) is defined as the duration of time between the onset of symptoms in an index case and the onset of symptoms in a secondary case. It is composed of the infectious period and the incubation period and is incorporated into transmission models as the 'generation time' (average time between the infection of an infector and the infection of their infectees). Estimates will be virus, host and methodology (e.g.

laboratory v clinical diagnosis, management of confounders) specific. Estimates for influenza range from 1-2 days (1918 pandemic (Sertsov *et al*, 2006)), 2-3 days [2009 pandemic (Boelle *et al*, 2011)] and 3-4 days (seasonal influenza in France (Viboud *et al*, 2004)). Cowling *et al* estimated an SI of 3.6 in Hong Kong during 2007 and, based on estimates that the incubation period of influenza is 1.4 days (Lessler *et al*, 2009), they suggest that the infectious period may be around 2 days (Cowling *et al*, 2009b). This is backed up by data generated during the 2009 pandemic. Donnelly *et al* observed an SI of 2.9 days and estimated that 82% of household transmissions occurred with 2 days of illness in the index case (Donnelly *et al*, 2011). France *et al* (using slightly different methodology) derived an estimate for the serial interval of 3 days in a school based outbreak in New York. They noted that 50% of secondary cases occurred within 3 days of illness onset in the index case giving an infectious period of 1.6 days in this group (France *et al*, 2010).

2.1.3 Secondary Attack Rate

The secondary attack rate (SAR) is the proportion of household contacts in whom illness occurs after the onset of symptoms in an index case.

Traditionally SAR has been used to measure household transmission but it is recognised that this can be difficult to do because it is hard to separate out true secondary infections from co-primary, tertiary and community acquired infections (Kemper 1980). The term 'subsequent attack rate' has been proposed as an umbrella term to account for these issues (Cannell *et al*, 2008) while others have attempted to account for confounding of the true SAR through the use of modelling (Longini *et al*, 1982; Cauchemez *et al*, 2009a; Donnelly *et al*, 2011).

Numerous estimates of the SAR for influenza have been derived from empiric household data and they range from 8-45% (Longini *et al*, 1982; Viboud *et al*, 2004; Cauchemez *et al*, 2009a; Yang *et al*, 2009; Cowling *et al*, 2010a; France *et al*, 2010; Papenburg *et al*, 2010). However, considerable variation exists in both the circumstances and methodologies used to generate them e.g. virus subtype, social structure, population immunity, environmental factors, how cases were recruited and defined, number of households observed, % of household members who were children and statistical methods employed.

2.1.4 Utility of epidemiologic data

Data that describe the epidemiology of influenza transmission provide a fascinating insight into the dynamics of transmission and serve to highlight the considerable variation that occurs between outbreaks. Crucially, these data can be used to plan and assess the potential effectiveness of a variety of infection control strategies;

- R_0 can be used to estimate attack rates and the speed of spread of infection nationally and internationally. It is possible to incorporate these data into models that predict the effectiveness of specific mitigation strategies, e.g. antivirals, travel restrictions, school closure and vaccination (Ferguson *et al*, 2006; Yang *et al*, 2009).
- Knowledge of the SI of a disease can help inform infection control policy at an individual level. Diseases with a short SI (e.g. influenza) can be difficult to control as there is little time to prevent onward transmission whereas it is easier to interrupt the spread of infection caused by diseases with a longer SI (e.g. SARS). Both pharmacological interventions (e.g. antivirals) and non-pharmacological interventions (e.g. hand hygiene, face masks) used to combat the spread of influenza should be implemented as early as possible. Data showing

that the infectious period for influenza is usually no longer than 2 days are used to determine advice on self isolation (CDC 2009a). The SI also has important consequences for research into interventions; if interventions cannot be deployed early enough any effect might be significantly underestimated.

- Several authors have attempted to estimate the origin of infection in household members; Yang et al estimate that 30-40% occur within the household and 20% in schools (Yang *et al*, 2009); Ferguson et al estimate that 30% occur in households, 33% in the general community and 37% in schools and workplaces (Ferguson *et al*, 2006) whilst Donnelly et al estimate that 23% of infections arise outside of the household (Donnelly *et al*, 2011). Using data from an epidemic in 1977-78, Longini et al estimated that the probability that an individual would become infected via a community source was 13% compared to 15% for a household source. Again, these data can be used to help select the most appropriate intervention strategies.

An important issue is the unpredictability of the transmission dynamics of the next pandemic. Models are based on assumptions and include estimates such as R_0 and the effectiveness of antivirals to reduce an individual's infectious period. Whilst estimates can be made, true values are only ever known after the event and it requires considered judgement to implement policies based on assumptions that could have a major impact on the well being of populations in real time.

Epidemiologic data also pose some intriguing questions about the transmission of influenza. Whilst it is clear that it can be a highly infectious virus, the SARs often observed and calculated (usually in the region of 25%) are surprisingly low. Furthermore, most households do not see

multiple cases. Mann et al found that 73% of infected family members were actually index cases (Mann *et al*, 1981), i.e. infection did not result from contact with an ill patient in a household whilst Hope-Simpson in a study spanning 8 years noted that 70% of households only had one influenza case (Hope-Simpson 1979). As a comparison SARs of 58%, 70% and 71% have been observed for rhinovirus, measles and varicella respectively (Fox *et al*, 1975; Narain *et al*, 1989; Seward *et al*, 2004). However, there are instances where SARs for influenza have appeared to be greater than 50% (Blumenfeld *et al*, 1959; Moser *et al*, 1979). This suggests that considerable variation in the transmission of influenza infection from sick to well people can exist.

In an attempt to explain these observations, Cannell et al propose that infected individuals are either good or bad transmitters, with a minority of good transmitters being responsible for spread of most infection (Cannell *et al*, 2008). This might also explain some of the variations seen in R_0 , SIs and SARs (as findings could depend on the proportion of good transmitters in a given population) and the fact that epidemics seem to end so abruptly (as a minority population of immune susceptible good transmitters are used up). There is good evidence to show that heterogeneity of infectiousness exists for a number of diseases. The concept of super-spreading, transmission of directly transmitted infections (e.g. measles, influenza and SARS) to an unusually large numbers of secondary cases from a source case, has been advanced by several authors (Woolhouse *et al*, 1997; Lloyd-Smith *et al*, 2005).

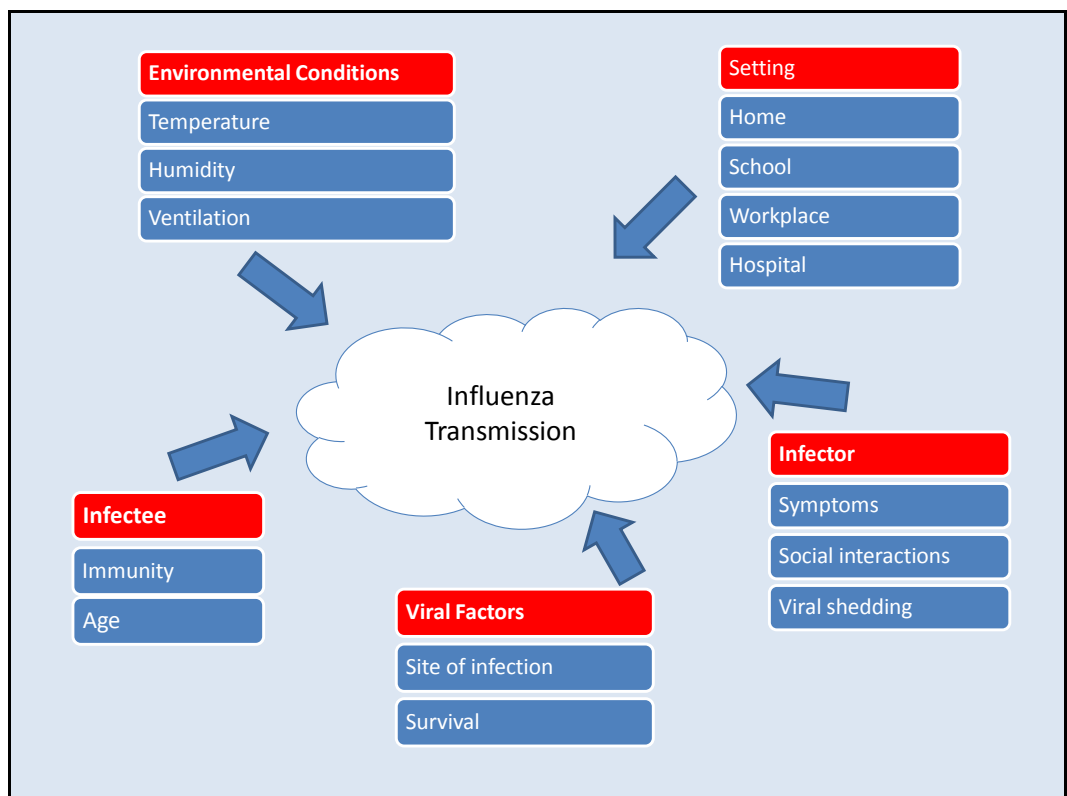
2.2 Factors Affecting Person-to-Person Transmission

Transmission of an infectious disease is the process by which an infectious organism moves from one host to another and causes disease. There are many factors which contribute to and influence this process and to appreciate them one must first understand the basic pathophysiology of a disease process.

Influenza replicates in epithelial cells throughout the respiratory tree (both upper and lower tracts) (Zambon 2001). Human viruses preferentially bind to cell surface receptors (sialyloligosaccharides) terminated by a N-acetylsialic acid linked to galactose by an $\alpha(2,6)$ -linkage (Rogers and Paulson 1983). The predominance of these receptors in different tissues reflects the tropism seen, e.g. $\alpha(2,6)$ are found mainly in the human respiratory tract (Shinya *et al*, 2006). As a result both virus entry and exit in humans occurs through the respiratory tract i.e. mouth and nose. Virus is released from a host during events such as coughing and sneezing which produce a 'respiratory spray' of different sized particles on which virus travels. It has also been shown the particles are released during talking and breathing (Papineni and Rosenthal 1997; Xie *et al*, 2009). Virus gains entry to a new host via respiration (droplet nuclei) and/or inhalation (droplets and droplet nuclei) and/or direct contact (droplets) and/or indirect contact (settled droplets). From here the target epithelial cells can be reached. The potential of the conjunctiva to mediate transmission of human influenza viruses remains uncertain (Weber and Stilianakis 2008) though data from tropism experiments with pandemic H1N1 (Chan *et al*, 2010b) and outbreaks of avian H7 viruses in humans that are marked by conjunctivitis confirms the presence of $\alpha(2,3)$ receptors in the eye (Olofsson *et al*, 2005). There is very little evidence to

suggest that the faecal-oral or waterborne route of transmission occurs in humans, in contrast to transmission that occurs amongst birds (Webster *et al*, 1978; WHO 2007). By considering the transmission pathway outlined above we can see that factors related to the virus, the environment and the host all contribute to transmission (Figure 2.1). To formulate and implement effective influenza control measures such as personal hygiene, social distancing and infection control it is critical to understand the above factors as each of these in turn can influence the route(s) of transmission that are active.

Figure 2.1: Factors that affect influenza transmission.



2.2.1 Viral Factors

The ability of different viral subtypes to infect and replicate in cells in the human respiratory tree varies. This has been attributed to differential binding of viruses to sialic acid receptors in the respiratory tree, and has been used to explain the observation that human infection with avian H5N1 viruses appears to predominate in the LRT and why human to human transmission is hardly seen (Shinya *et al*, 2006), though some studies suggest a more complex picture (Nicholls *et al*, 2008). Through the use of animal models a better understanding of the viral determinants of transmission is developing. The HA and polymerase proteins seem to be important, though the variety and interplay of traits is intricate, some seeming to hinder transmission whilst others permit it through different routes. (Belser *et al*, 2010).

2.2.2 Environmental Conditions

The ability of virus to survive outside the human body is critical for infection transmission to occur. For example, virus must survive in air or on surfaces to be able to transmit via aerosols and indirect contact respectively. Variables such as temperature and humidity have been shown to affect this (Wells and Brown 1936; Loosli *et al*, 1943; McDevitt *et al*, 2010). Furthermore the influence of temperature and humidity has been demonstrated in transmission experiments between animals (Lowen *et al*, 2007; Lowen *et al*, 2008).

2.2.3 Human Factors (infector/infectee)

- Immunity

Levels of immunity (both humoral and cell mediated) to particular viral subtypes in a population may explain in part the different rates of infection seen. In seasonal influenza, the burden of illness typically falls upon the

young and the elderly, either because of deficient immunity or a susceptibility to complications of influenza infection (Barker and Mullooly 1980; Thompson *et al*, 2003; Mullooly *et al*, 2007; Pitman *et al*, 2007). Because pandemic viruses are antigenically novel, a lack of pre-existing immunity in a population will be expected. However, the 2009 pandemic was notable for the fact that a significant proportion (about a third) of the population over the age of 65 had cross reactive antibodies compared to $\leq 10\%$ in younger adults and very little in children (Hancock *et al*, 2009; Miller *et al*, 2010). Immunocompromised individuals are known to have a higher frequency of influenza infections (Kunisaki and Janoff 2009) and once infected they can shed virus for a prolonged period (Hayden 1997; Lee *et al*, 2009).

- Age

A recurring risk factor for transmission is young age; both young age of index cases (Viboud *et al*, 2004; Cauchemez *et al*, 2009a) and young age of contacts (Viboud *et al*, 2004; France *et al*, 2010; Papenburg *et al*, 2010) have been significantly associated with transmission. Viboud *et al* estimate that 40-48% of transmissions in a household are attributable to sick children (Viboud *et al*, 2004). Longini *et al* found that the probability of community infection was higher in households with children compared to those without suggesting that schools and nurseries play an important role in disease spread (Longini *et al*, 1982). In addition, a study that looked at social contact networks in young people found evidence of super-spreaders (Glass and Glass 2008). The importance of children in the spread of viral respiratory infections, including influenza, is widely recognised (Monto 2002; McLean *et al*, 2010) and may be explained by a number of factors including increased numbers of contacts, lower levels of immunity (Longini *et al*, 1988), increased duration and levels of viral

shedding (Frank *et al*, 1981; Welliver *et al*, 2001; Sato *et al*, 2005; Cao *et al*, 2009; To *et al*, 2010) and poor respiratory and hand hygiene.

- Viral shedding

An individual who sheds more virus (via droplets and aerosols) into the environment or who's duration of viral shedding is longer than average could be more infectious. The dynamics of viral shedding are important factors, highly relevant to estimates of the period of infectivity and to therapeutic management. Shedding is determined by measurement of the quantity of virus recoverable from the patient's nasopharynx. It is well established that viral titres in nasopharyngeal samples taken from adults are proportional to symptom severity and decline steadily from symptom onset (Boivin *et al*, 2000; Treanor *et al*, 2000; Lee *et al*, 2009; Lau *et al*, 2010; Ng *et al*, 2010). Studies of patients infected with influenza A in the community show that the mean duration of viral shedding [detected by polymerase chain reaction (PCR)] for seasonal influenza A viruses is 5-6 days from symptom onset (Lau *et al*, 2010; Ng *et al*, 2010) compared with culture methods which are normally negative by Day 6 (Boivin *et al*, 2000; Lau *et al*, 2010). It is also well documented that children, patients with chronic illnesses, hospitalised patients and those who are immunocompromised can shed live virus for longer periods (Frank *et al*, 1981; Sato *et al*, 2005; Leekha *et al*, 2007; Lee *et al*, 2009). Use of systemic steroid by patients with asthma or COPD can also prolong shedding (Lee *et al*, 2009).

Whilst PCR is almost certainly a more sensitive measure of viral shedding because it detects both viable and non-viable virus, it is not possible to distinguish between them. Thus it can only illustrate the potential for viable (infectious) virus to be present. As this is the most common method

of assessing viral shedding in clinical practice, its interpretation can create problems in deciding whether a patient is infectious or not. However, there have also been difficulties in deciphering studies looking at live virus because of the range of techniques used for detection (cell lines, animal models and human beings) and variation in sensitivities between and even within such methods, e.g. a human infective dose is likely to differ from a tissue culture infectious dose.

Despite these issues, knowledge of viral shedding patterns can be useful in determining the optimal impact of a range of infection control measures;

- Self-isolation – Based on the duration of viral shedding and estimates of the infectious period, guidelines suggest that infected individuals should self-isolate themselves until they have been free of fever for 24 hours whilst hospitalised individuals should be isolated for 7 days from the onset of illness (CDC 2009a). Self-isolation means avoiding unnecessary contact with others, particularly individuals who might be at high risk of becoming infected or of developing complications from infection. This often means taking time off work or school. This advice could potentially have a big impact on populations, for example the economic impact of lost work days. Therefore, a careful balance must be struck between safety and practicality and this may well differ between settings.
- Antivirals – Antiviral drugs have been shown to reduce both the amount and duration of viral shedding and earlier treatment (within 48 hours) leads to a bigger effect (Nicholson *et al*, 2000; Treanor *et al*, 2000; Aoki *et al*, 2003; Baccam *et al*, 2006; Lee *et al*, 2009; Li *et al*, 2010; Ling *et al*, 2010). Antivirals are also associated with reduced symptom severity and accelerated symptom resolution (Nicholson *et al*, 2000; Treanor *et al*, 2000). Despite these findings however, an

effect on infectiousness has been difficult to prove (Halloran *et al*, 2007; Ng *et al*, 2010).

An important feature of infection in some individuals is that they shed virus but do not experience symptoms. This may happen early in the course of infection before symptoms begin (pre-symptomatic shedding) or exist throughout the course of an infection if symptoms do not develop (asymptomatic shedding). Such individuals may not seek treatment or self-isolate and therefore may be an important group. Models have typically assumed that asymptomatic or subclinical infections make up 33-50% of all infections (Longini *et al*, 2004; Ferguson *et al*, 2006) though empiric data obtained during the 2009 pandemic showed asymptomatic infection rates of 8-18% (Lau *et al*, 2010; Papenburg *et al*, 2010; Suess *et al*, 2010; Cook *et al*, 2010) with a subclinical rate of 25% (Lau *et al*, 2010). Lau *et al* estimated that 1-8% of infectiousness occurred prior to illness onset (Lau *et al*, 2010). However, the amount and duration of viral shedding from asymptomatic patients can be low (Cowling *et al*, 2010a; Lau *et al*, 2010) and it remains to be shown that asymptomatic individuals effectively transmit influenza (Patrozou and Mermel 2009).

2.2.4 Setting

Each setting reflects a specific combination of source cases, susceptible individuals and environmental conditions. Transmission within a household is commonly measured; attack rates in the region of 20% are seen and are determined by the size of the household, the presence of children and the number of susceptible occupants (Longini *et al*, 1982). Schools are often thought of as being focus for spread in the community. This is fuelled by high attack rates and infectiousness of children (Cauchemez *et al*, 2009a; Chen and Liao 2008). School closure can be an effective

mitigation strategy (Cauchemez *et al*, 2009b). Closed environments such as nursing homes and military camps are often reported to have attack rates greater than 40% (Balkovic *et al*, 1980; Patriarca *et al*, 1987; Earhart *et al*, 2001; Liu *et al*, 2009) (though one must be aware of reporting bias) and high attack rates have been reported in healthcare facilities (Horcajada *et al*, 2003). Travel can be responsible for the spread of viruses across large distances and the mode of travel can be associated with infection risk; outbreaks have been reported on aeroplanes, buses and trains (Moser *et al*, 1979; Han *et al*, 2009; Baker *et al*, 2010; Cui *et al*, 2011).

2.3 Routes of Transmission

Despite the fact that influenza has impacted on human health for at least several centuries (Potter 2001) and that the virus was first identified in humans in 1933 (Smith *et al*, 1933), remarkably little is known definitively about its modes of transmission. Thus, important health policy and infection control issues remain unresolved. These shortcomings have been exposed in national and international pandemic preparedness activities over recent years and during the 2009 pandemic itself.

2.3.1 Definitions

One of the difficulties that arises in the reviewing the literature on influenza is the inconsistency and variety of terms that are used to refer to the modes of transmission. Traditionally the standard definitions used by the Centers for Disease Control (CDC) to describe modes of infectious disease transmission (Garner 1996) have included;

- Direct Contact – transmission via direct physical contact; for example a kiss.
- Indirect Contact – transmission via an intermediate object such as a fomite or a hand.
- Droplet – droplets are particles $>5\mu\text{m}$ and are generated from the respiratory tract. They act like ballistic particles and hence some view them as a form of direct contact.
- Airborne – transmission by bioaerosols; particles $<5\mu\text{m}$ (droplet nuclei) that can remain suspended in air, travel long distances ($>6\text{ft}$) and deposit in the lung.

Airborne transmission has generally been used to refer to infections that spread over long distances, for example tuberculosis. It is only droplet nuclei in aerosols that remain suspended for prolonged periods in the air and can travel over long distances ($>2\text{m}$) but some confusion can arise because; i) there is no absolute cut-off between droplet nuclei and droplets, particles lie on a continuum with larger particles tending towards droplet behaviour. Furthermore, all aerosolised particles are dynamic, that is they change size as water is exchanged (taken up or released) with the atmosphere and this is dependent upon factors such as humidity, temperature and airflows; ii) droplets could be considered to be airborne as they travel through the air (although only for a short period of time and over short distances) and iii) droplet nuclei can transmit infection over short distances as well as long. In fact, because droplet nuclei are more concentrated nearer their source, they are more likely to transmit over short distances than long.

The following terms which are based on working definitions used by Weber and Stilianakis in a review of influenza transmission (Weber and

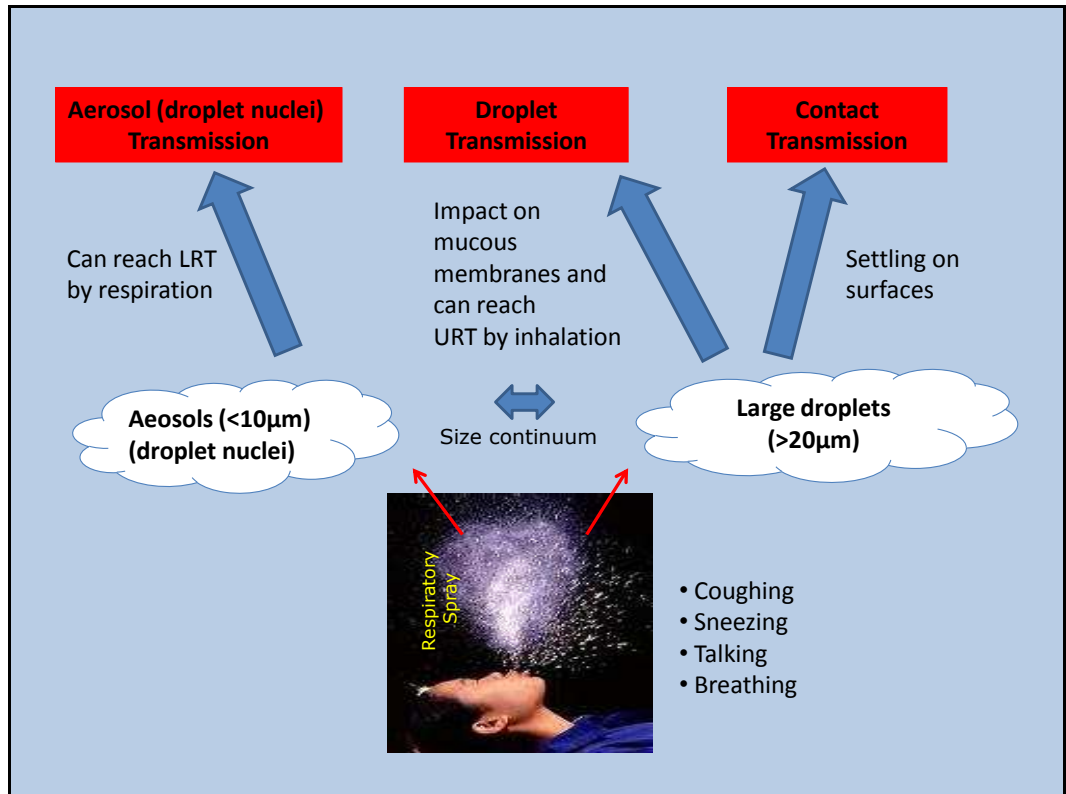
Stilianakis 2008) and those used at a CDC workshop on influenza transmission (CDC 2010a) will be used in this thesis (Figure 2.2).

- Droplet transmission: Transmission of influenza through the air by droplet particles ($>20\mu\text{m}$) emitted by an infected host (e.g. by coughing) which deposit directly on to mucous membranes. It is likely that an infectious virus particle will reach its target cell by inhalation more commonly than by direct contact.
- Aerosol transmission: Transmission of influenza through the air by droplet nuclei ($<10\mu\text{m}$) which can be respired. Particles penetrate proximal airways to reach the lung and can initiate infection there. The behaviour of particles $\geq 10\mu\text{m}$ and $\leq 20\mu\text{m}$ is difficult to predict; they are considered to lie in a 'grey zone'.
- Contact transmission: Comprising direct and indirect contact (as defined above)

A sound understanding of the basic science of influenza transmission is key to developing evidence-based policies for infection prevention and control. At present opinions are sharply divided on the importance of aerosol versus droplet transmission (Tellier 2006; Brankston *et al*, 2007). The uncertainty about the importance of different mechanisms of influenza transmission and the best means to prevent spread was reflected in the diverse approaches adopted by different countries in response to the 2009 pandemic. For example, the UK in line with WHO, recommended droplet as opposed to aerosol infection control precautions (i.e. SFMs rather than respirators) for HCWs for most close contact with patients (Department of Health 2009; WHO 2009d), whereas US (CDC 2009c; CDC 2009e) and French (Secretariat General de la Defense Nationale 2009) guidance recommended respirators for all forms of close contact. At present there is little in the way of firm evidence with which to formulate guidance for

healthcare workers as to the level of risk reduction provided by the different types of protective equipment.

Figure 2.2: Routes of influenza transmission.



Footnote: The figure shows the release of a respiratory spray, its composition in terms of particle sizes and the routes of transmission that may result. LRT = Lower Respiratory Tract, URT = Upper Respiratory Tract

2.4 Influenza Transmission Evidence Base

The evidence base on influenza transmission is largely derived from six core categories of study:

1. Studies assessing influenza virus deposition and survival in the environment that inform the biologic plausibility of the proposed routes of transmission.
2. Studies examining the epidemiology of disease in hospitals, nursing homes and other closed or semi-closed settings. From these data, inferences are drawn about modes of transmission that could have produced the pattern of disease observed.
3. Prospective pharmaceutical and non-pharmaceutical intervention (NPI) studies in the setting of natural infection.
4. Human influenza challenge studies; infection, initiated by a number of routes, and subsequent patterns of viral shedding have been described for experimentally infected individuals in a relatively small number of studies.
5. Animal models of transmission; information generated from experimental studies in different animal models can provide useful insights, however, any extrapolation to humans relies on assuming transmission mechanisms and behaviours are similar in humans and other animals.
6. Modelling has been used to explore the relative contributions that each route of transmission may have.

The evidence relating to each category of evidence is presented below and table summaries of reviewed studies can be found in Appendices 2.1–2.6.

2.5 Biologic plausibility for the proposed routes of transmission

2.5.1 Contact transmission

For contact transmission to occur; i) viable virus is released from a host; ii) virus must survive for a period of time on hands or fomites; and iii) an infectious dose of virus must be delivered to a site where infection initiation can occur. There exists significant heterogeneity in the design and methods of studies that have examined virus survival and it is difficult to draw unifying conclusions. Variations take the form of; virus strains examined, concentrations of inocula used, manner of inoculation, populations studied, environmental conditions, sampling methods and detection techniques. Efficient sampling and detection are vital as viable virus is easily lost during experimental manipulations. This is likely to pose a greater challenge outside the setting of controlled laboratory experiments. Furthermore, whilst laboratory based studies are useful for defining parameters of what may be possible, the relationship between laboratory studies and what happens in 'natural' conditions is difficult to judge.

Despite some limitations, there is good evidence to confirm the ability of influenza to remain viable on fomites (Bean *et al*, 1982; Thomas *et al*, 2008; Shaman and Kohn 2009). Survival on hard non-porous surfaces where drying times are longer than those of porous surfaces usually extends well beyond 24 hours. However, the ability to survive does not necessarily equate to the ability to infect and whilst studies have shown that virus can be found on fomites, particularly around children (Boone and Gerba 2005; Bright *et al*, 2010; Simmerman *et al*, 2010), none have found live virus or established whether infection transmission from such fomites occurs.

The importance of environmental conditions is illustrated by a study which demonstrated that influenza virus survival is affected by temperature, relative humidity (RH) and exposure time after being deposited on a stainless steel surface; viral inactivation increased with rising temperature (55 to 65°C) and RH (25 to 75%) (McDevitt *et al*, 2010).

The microenvironments of viruses are also important. Thomas *et al* studied virus survival on banknotes; survival time was prolonged when viruses were inoculated on to banknotes after being mixed with respiratory mucous (Thomas *et al*, 2008). At the patient level, inter- and intra-patient variation complicates the issue; patients will shed virus at different titres during the course of their illness, some will shed more than others and environmental conditions may differ.

There is evidence that virus can survive on hands for at least 5 minutes. In a study by Grayson *et al*, the hands of 20 volunteers were contaminated with virus. After 2 minutes, a 3-4 log reduction in virus (as measured by PCR and culture) was seen, though interestingly little further reduction was seen after an hour (Grayson *et al*, 2009). Thomas *et al* contaminated the fingertips of six volunteers. In the first part of the study the effect of time was assessed; virus was detected on 100%, 28-44% and 11% of fingertips after 1, 5 and 30 minutes respectively. It was then shown that bigger volumes of inoculums led to more virus being detected at 15 minutes and that if the viral inoculums were spread on the fingertip (rather than being left as a drop) survival was less (Thomas *et al*, 2010). Bean *et al* showed that virus can be transferred from deliberately contaminated fomites to hands but that within 5 minutes titres had fallen by at least two logs (Bean *et al*, 1982). Although survival on hands appears significantly reduced compared to some fomites this may not be

significant if hands frequently 'collect' virus and then deposit it on mucous membranes [face touching has been shown to occur at a rate of 15.7 events per hour (Nicas and Best 2008)]. As part of a randomised trial in Thailand to investigate hand hygiene, the hands of 149 infected patients were swabbed (on Day 3 of illness). Fifteen out of 90 (16.7%) index cases and 1 out of 59 (1.7%) household contacts were positive by PCR. Only one (0.7%) was culture positive (Simmerman *et al*, 2010). Another obstacle is that the infectious dose of influenza transmitted in this way is not known. Even if viable virus is detected, is enough of it present to cause infection?

Based on their data and making certain assumptions (e.g. a 50% human infectious dose = 30-127 TCID₅₀ and the transference of a 0.01-0.02mL inoculum from surface to hand) Bean *et al* conclude that a person shedding large quantities of virus ($>10^{5.0}$ TCID₅₀/mL) could transmit infection via stainless steel for 2 hours and via tissues for a few minutes (Bean *et al*, 1982).

Despite the above, there is currently little direct evidence to confirm that infection transmission can occur via the contact route.

2.5.2 Droplet Transmission

This route of transmission is reliant on close contact so that a droplet carrying infectious virus, expelled from an infected individual, comes into contact with the respiratory tract of a susceptible individual. It is mediated by large droplets (considered to be particles $>20\mu\text{m}$ and detected up to a size of $1000\mu\text{m}$) which behave like ballistic particles after being generated by activities such as coughing and sneezing (Nicas and Sun 2006; Weber and Stilianakis 2008; Gralton *et al*, 2010). It has been shown that the vast majority of pathogens emitted in a cough will be carried by large droplets

(Lidwell 1990; Nicas *et al*, 2005; Chao *et al*, 2009). The distance these particles travel is determined by their initial velocity, their terminal velocity and gravitational acceleration. It has been estimated that particles >150µm can travel >60cm (Nicas and Sun 2006). So, although the majority of droplets expelled during a cough or sneeze will settle to the ground quickly and not reach a susceptible host, they remain important as any droplets that do reach target cells can carry a high pathogen load. Droplets reach respiratory epithelial cells via direct contact or inhalation; the latter is perhaps more likely to deliver an infectious particle than contact as the probability that a cough or sneeze is perfectly directed so that particles land directly on epithelial cells is small (Nicas and Sun 2006). Initiation of infection following the inhalation of particles is dependent on several factors such as infectious dose [thought to be higher in the URT than the LRT (Tellier 2006)], nose or mouth breathing, tidal volume, breathing rate and timing so that an inspiratory breath in a susceptible contact occurs immediately after particle generation by an infected case. So, whilst the basic concept of droplet transmission may at first be readily accepted, the constraining factors mentioned have actually led some to consider it a rare event (Atkinson and Wein 2008).

2.5.3 Aerosol Transmission

Bioaerosols (aerosols that contain living organisms) can be generated by actions such as coughing, talking and breathing and may transmit infection on being inhaled. Galton *et al* propose that the spread of infection by aerosolised particles is dependent on; the clinical manifestation of disease, the site of infection, the presence of pathogen and the type of pathogen (Galton *et al*, 2010). The process of disease transmission via aerosols has been reviewed in depth (Nicas *et al*, 2005; Tang *et al*, 2006; Galton *et al*, 2010). For influenza viruses to be

transmitted from human to human by the aerosol route they will need to be emitted from an infected individual in particles that can then be inhaled to the LRT of the exposed individual so bringing the virus into contact with target cells. In addition, the concentration of these particles must be high enough to deliver an infectious dose. Furthermore, the virus must survive the stresses of aerosolisation and be able to survive in the air for long enough to permit transmission. The behaviour of a virus within aerosol particles depends on the behaviour of the particle (aerosol physics) and the reaction of the virus to being in aerosol form (aerosol stability).

- Bioaerosol production

In a single sneeze, the total volume of particles $\leq 10\mu\text{m}$ has been estimated at 1.2×10^{-5} mL (Nicas *et al*, 2005). Virus concentration from nasal washes has been shown to be as high as 10^7 TCID₅₀/mL (Murphy *et al*, 1973). Hence, a patient with a titre of 10^7 TCID₅₀/mL of nasal secretion would generate 120 TCID₅₀ in aerosols in a single sneeze (assuming that a sneeze equates to a nasal wash in terms of the amount of virus 'released'). Given that the aerosol Human Infectious Dose (HID₅₀) is 0.6 to 3 TCID₅₀ (Alford *et al*, 1966) a single sneeze could create a significant infectious aerosol (Tellier 2007).

Using data from Chao *et al* who examined particle size distributions generated by a cough (Chao *et al*, 2009), it can be shown that if patients have low titres of virus in respiratory secretions, the vast majority of virus will be present in large particles. However, if patients are excreting higher titres then the presence of virus in particles of $< 10\mu\text{m}$ is feasible. For example if the original titre is 10^7 TCID₅₀/mL then virus will be present in all particles $\geq 10\mu\text{m}$, but only in a fraction of particles $< 10\mu\text{m}$ (50% at $9\mu\text{m}$, 4% at $4\mu\text{m}$) when a patient coughs (Killingley *et al*, 2011b). This

could imply that aerosol transmission is more likely at the beginning of infection when patients typically excrete higher titres of virus and that children are important aerosol transmitters as they excrete higher titres of virus than adults.

Another obstacle to understanding the nuances of bioaerosol production is that individuals differ in the numbers and sizes of particles produced during breathing, coughing, sneezing and talking (Papineni and Rosenthal 1997; Edwards *et al*, 2004; Xie *et al*, 2009; Holmgren *et al*, 2010).

Differences can arise from a number of factors including behaviour/activities, viral shedding, symptoms and presence of co-infections.

- Virus survival in aerosols

There is evidence to suggest that virus can remain viable (and therefore infectious) in aerosols long enough to permit infection transmission (Weber and Stilianakis 2008). Studies performed over 40 years ago showed that artificially aerosolised influenza could be recovered from the air (by using infection in animals as a detection method) for up to 24 hours after release (Wells and Brown 1936; Loosli *et al*, 1943). Overall investigators have found that survival is prolonged at low RH and this has lent support to the idea that low RH in indoor environments during winter time promotes virus survival and transmission. However, methodological limitations to the reviewed studies should be noted. For example the size of aerosols used varied and the use of small particles (<3µm) may stress the virus to a higher degree than during natural generation which may lead to an underestimate of survival.

- Detecting bioaerosols

Despite the above, the detection of live virus in aerosols, released into the natural environment by humans (a key step to confirm plausibility) has not been shown before. However, the evolution of the materials and methods used to collect bioaerosols is contributing to progress in this field; a comprehensive review of methods was published in 2008 (Verreault *et al*, 2008). Contemporary efforts to detect influenza virus in aerosols have been successfully achieved by a number of groups, both in the laboratory (Blachere *et al*, 2007; Pyankov *et al*, 2007; Fabian *et al*, 2009a) and from around patients (Fabian *et al*, 2008; Blachere *et al*, 2009; Lindsley *et al*, 2010a). Fabian and colleagues developed a technique to look for influenza virus in the exhaled breath of infected patients. Patients were asked to directly breathe into a device that collects filtered samples and employs optical particle counting and airflow data. Influenza was detected by PCR in 4 out of 13 samples collected from patients confirmed to be infected (Fabian *et al*, 2008). Blachere *et al* have described the use of a two stage, cyclone-based bioaerosol sampler. Following aerosolisation of influenza virus they were able to successfully collect and detect virus (by PCR). At the same time collected particles were size fractionated allowing particles of a respirable size to be identified (Blachere *et al*, 2007). They went on to test the samplers, which can be worn by individuals, in medical care facilities in the US. Both stationary and personal samplers collected air particles containing influenza A virus (they were also able to detect Influenza B and rhinovirus in the second study) (Blachere *et al*, 2009; Lindsley *et al*, 2010a).

2.6 Outbreak Investigations

The primary function of an outbreak investigation is to instigate control and prevention measures, though the investigation itself can lead to opportunities to gain additional knowledge about the disease, for example spectrum of illness, transmission characteristics and incubation periods.

Influenza outbreak reports are extremely heterogeneous, each relates to a specific situation with a variety of key factors including virus strain, human hosts, environmental setting and environmental conditions.

Several reports are discussed in more detail below:

- A prospective observational study took place during the 1957/58 pandemic in the US which centred on a hospital in California that had a building where UV lights had been installed. One hundred and fifty patients resided in this unit, principally patients with tuberculosis and other chronic respiratory diseases. The UV lights were being used to disinfect the air of *M. tuberculosis*. Another hospital building housing 250 patients with respiratory disease but without UV lights served as a control. Serological assessments were done before and after the second wave of an epidemic that struck in January 1958. Both clinical illness and serologically confirmed infection rates were reduced in the patients housed under UV lights (2% v 19%) (McLean 1961). These data appear to make a compelling argument for effect of UV light on bioaerosols and suggest in turn that influenza is transmitted via aerosols (UV light cannot penetrate larger particles and will not affect virus associated with them). However, as the author acknowledges, this was not a controlled experiment and several confounders may have existed. For example, no mention is made of patient movements, length of stays and there are no descriptions of cases. In addition, the

environments on the two wards may have been different with respect to ventilation (e.g. airflows, open windows) and staff illness and movement between wards are not discussed in any detail. Because of these factors we cannot be sure that differences in attack rates seen were solely due to the effect of UV irradiation.

- An outbreak aboard a grounded aircraft that occurred in Alaska in 1977 is reported by Moser (Moser *et al*, 1979) with further information provided by Gregg (Gregg 1980). A total of 54 people were on board the aircraft at some point during its 4.5 hour grounding for a mechanical fault; 53 were followed up. The index case was a 21 year old female who was symptomatic with fever and cough. She was subsequently found to have seroconverted to an H3N2 influenza virus. 30 passengers and crew stayed on the aircraft for the entire time, this includes the index case who lay across two seats and did not move about the plane. The exposure time for others varied as passengers were allowed to leave the aircraft and wait in the terminal building. In total 38 (72%) people became ill; 8 out of 31 were culture positive and 20 out of 22 were serologically positive. Individuals with greater than 3 hours of exposure had an attack rate (AR) of 86% whilst less than 3 hours of exposure gave a 54% AR. This outbreak featured a single source of infection, a high AR and a small, enclosed space in which the ventilation system had been switched off. The authors concluded that "exposure to large aerosols" was likely responsible for infection transmission. Large is not defined in this instance but it seems most likely that they are referring to aerosol transmission as opposed to droplet. We may speculate that the index case was emitting a large bioaerosol load (a so called super-emitter); this might help explain the high AR. However, it is difficult to completely exclude the droplet and

contact routes of infection; patients were able to move around the aircraft and thus close proximities to the index case could have occurred though the index case herself remained stationary and is not reported to have had direct contact with anyone else.

- Morens et al reported on an outbreak in a nursing home residence in Hawaii that occurred in 1989 (Morens and Rash 1995). Each of the home's 12 rooms contained one to four beds. Among 39 residents, 11 became clinically ill (28%); of these five were bedfast (11 were bedfast in total); six were virologically confirmed to have influenza and six died (three of whom were virologically confirmed). Infection control measures were instituted but only after more than 2 weeks from the onset of symptoms in the first case. An outbreak investigation revealed the following ARs in residents;
 - Those needing skilled nursing care 34% vs. those needing intermediate care 10%.
 - Bedfast 45% vs. non bedfast 21%.
 - Tube fed or frequently suctioned 38% vs. others 13%.
 - Those who were mobile and socialised with other residents had lower ARs than bedfast or tube fed patients.

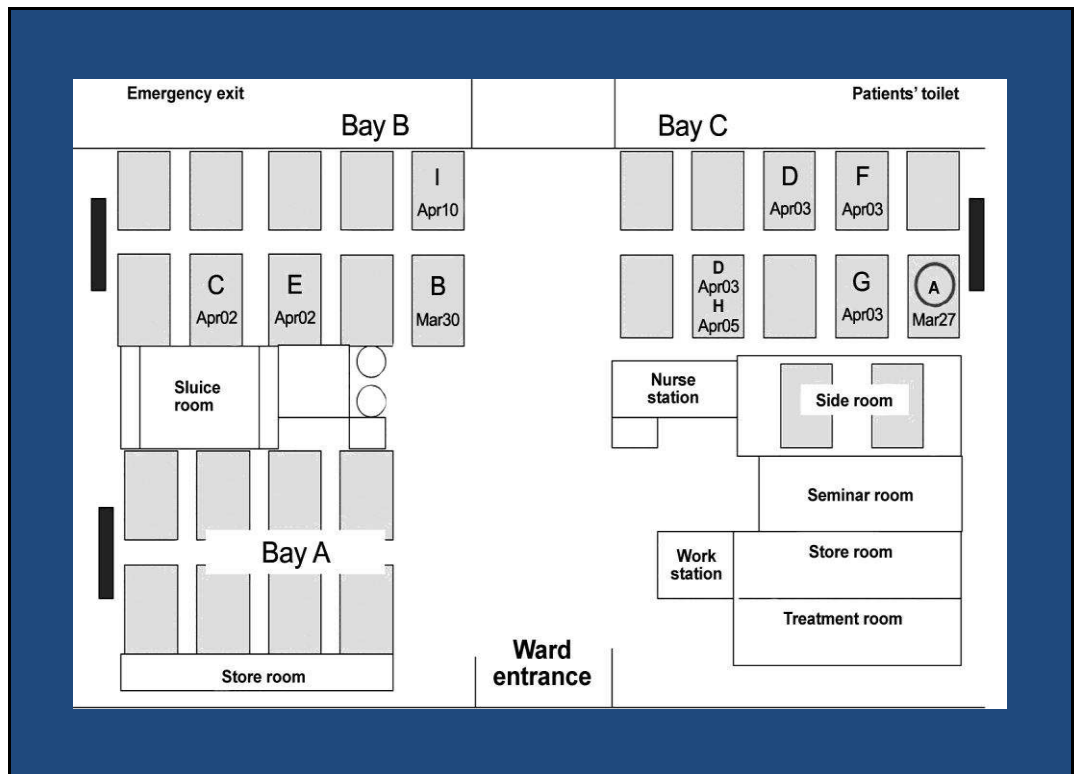
In addition, it was noted that individual staff were in contact with more nursing home residents than was normal. The authors commented that the 'spatial and temporal patterns of onset not typical of airborne spread' and that 'we suspect staff spread virus by hands or fomites'. No staff illness was reported so it is unlikely that they acted as primary vectors. It is impossible to exclude either droplet or aerosol spread in the scenario described but the unusually high levels of patient contact and a lack of strict infection control procedures do appear significant.

- An outbreak of H3N2 influenza occurred on a neonatal unit during an epidemic in Ontario, Canada in 1998 (Cunney *et al*, 2000). Of 54 neonates present in the unit over 18 days, 19 (35%) were confirmed cases though only six were symptomatic. 16% of staff reported illness during the outbreak. Risk factors for infection in neonates were being a twin [odds ratio (OR) = 7] and being mechanically ventilated (OR = 6.2). The unit was very busy over the period with >100% bed occupancy. The risk factors above seem to indicate that close contact is important. Perhaps parents were responsible for passing infection between twins, and nurses who have increased contact with ventilated children, also acted as secondary vectors. In the report the authors comment that they discovered that “ventilator tubing was being changed in a manner that produced aerosols”.
- A tour group comprising 30 people travelled to China for a 4 day trip in June 2010. The index case was a female tourist who developed symptoms on the first day of the trip. The tour included time spent on a bus and two aeroplane flights. Confirmed secondary cases of A(H1N1)pdm09 included nine tour group members and one aeroplane passenger who was not part of the tour group (this passenger was seated within two rows of the index case). The investigators reported that talking to the index case for greater than 2 minutes (at a distance of less than 2m) was associated with an AR of 56%; nobody who did not talk to the index case became ill. Furthermore, talking for greater than 10 minutes increased the chances of becoming ill by five times compared to talking for between 2-9 minutes (Han *et al*, 2009). It seems that close proximity to the index case was necessary for transmission, droplet or contact transmission are certainly possible. The authors state that there was “no evidence of airborne

transmission". Certainly, there is little evidence for long range transmission but the possibility of short range aerosol transmission (SRAT) is overlooked, especially when one considers that talking and normal breathing can generate aerosols (Edwards *et al*, 2004).

- A retrospective cohort study concerning a school group (n=24) that travelled to Mexico and returned home on a flight from Los Angeles to New Zealand in April 2009 is reported by Baker (Baker *et al*, 2010). A general practitioner in NZ identified cases of ILI amongst members of the group soon after their return and this led to an investigation to assess disease transmission during the air flight home. During the flight 12 cases reported symptoms; nine were virologically confirmed with A(H1N1)pdm09, three were suspected. A post-flight case was defined as illness appearing within 3.2 days of the airplane landing. At risk for in-flight infection were 102 passengers in rear section of plane; 97 (95%) of these individuals were contacted and nasopharyngeal swabs were collected from 26. Four post-flight cases were identified; of these two were deemed probable, one possible and one inconclusive for in-flight infection. The overall risk of infection in the rear section of the plane was 1.9%. For 57 passengers who were seated within two rows of a symptomatic case the risk was 3.5%. The authors conclude that the "mode of transmission cannot be established, all are possible including SRAT". Long range transmission was not evident.
- An outbreak investigation in a Hong Kong hospital in 2008, that paid special attention to airflows is described by Wong (Wong *et al*, 2010). The setting was a 30 bedded medical ward that housed 59 patients and 29 HCWs over the course of the outbreak. The ward was composed of three bays (A, B and C) and a side room (Figure 2.3).

Figure 2.3: Layout of the outbreak ward and the locations of affected patients.

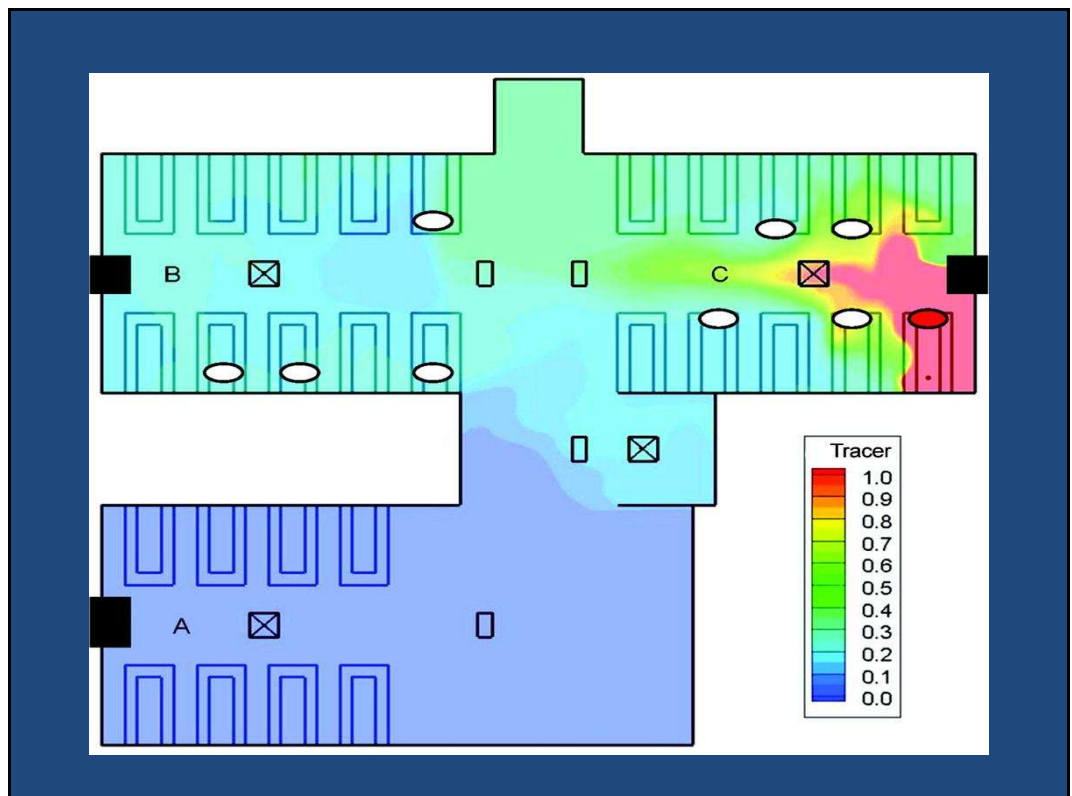


Footnote: Patient A (circled) was the index case. Dark-colored blocks represent high-efficiency particulate absorbing (HEPA) filters placed at the wall end of each ward bay. Dates of symptom onset are stated for all infected patients. Patient D had been staying at two bed locations (front row then back row). Reproduced with permission from (Wong et al, 2010).

The index case had chronic obstructive pulmonary disease and received non-invasive ventilation for 16 hours on the ward (Bay C) beginning on March 31st. Influenza H3N2 was subsequently diagnosed. Nine inpatients were confirmed as secondary cases and two HCWs developed symptoms but were not virologically confirmed. All cases received oseltamivir within 24hrs. The overall patient AR was 13.6%; ARs in Bays C, B and A were 20%, 22.2% and 0% respectively. The risk of infection was found to be highest on 31st March and 1st April. A variety of devices were in operation that affected airflows on the ward; i) air conditioning was provided by a system that had outlets at

ceiling level in each bay. Return air grills were located in the ward corridors; ii) air purifiers were also located in each bay; in bays A and B the fan setting was low but in Bay C the fan setting was on medium. This resulted in a net flow of air from Bay C into the corridor and towards Bay B (Figure 2.4).

Figure 2.4: The spatial distribution of normalized concentration of hypothetical virus-laden aerosols (modelled as gaseous tracer) in the outbreak ward.



Footnote: The tracer was released at a height of 1.1m. All HEPA filters were assumed to function with 100% filtration of the modeled droplet nuclei. The three HEPA air purifiers are shown as black boxes, the four air conditioning inlets are shown by a square with an X, and the four returns are shown as a small rectangular filled box. Affected patients are represented by white ovals (the index patient is marked as a red oval). Reproduced with permission from (Wong et al, 2010).

The outbreak was temporally related to an aerosol generating procedure involving the index case and imbalanced airflow on the ward. The authors state that droplet and contact spread cannot entirely explain all instances of infection transmission. They cite as evidence the epidemic curve which supports a point source for the outbreak, the spatial distribution of secondary cases seen and the fact that close contact transmission was minimal as there was little patient interaction and little evidence that HCWs acted as vectors. This study presents a unique set of circumstances and convincing evidence for the presence of aerosol transmission.

The very nature of an outbreak means that conditions are not formally controlled in any way making it very difficult to draw firm conclusions about specific risk factors for, and routes of transmission. This leaves us to interpret findings based upon observations only. Some studies do describe situations akin to a control and intervention group, whilst others describe specific environmental factors that existed which may have influenced the spread of infection. Repeated observations of outbreaks in closed settings show that as population densities increase, ARs also increase. This implies that short range transmission, by whatever route, is important. Long range transmission is a rare event; however, this does not mean that aerosol transmission can be discounted. The concept of SRAT is often overlooked with the consequence that transmissions that have been seen to occur through close contact are put down to either droplet or direct contact spread. In the studies reviewed no routes of transmission can be completely excluded, circumstances related to the environment and individuals involved will dictate which route(s) predominate. For example, the reports by Moser, Mclean and Wong appear to support the existence of aerosol transmission because of circumstances that favoured this route.

2.7 Prospective intervention studies in the setting of natural infection

Non-pharmaceutical interventions (NPI) such as hand hygiene and face masks are recognised by WHO as being potentially useful to reduce the transmission of influenza between people (Bell 2006b). Such interventions may be able to tell us something about transmission routes because they act by disrupting one or more of them. For example if hand hygiene is shown to reduce illness rates then it implies that the contact route of transmission is significant and if wearing a SFM reduces illness rates then either the contact and/or droplet route(s) are important (a SFM will act as a barrier to both).

Three systematic reviews (Rabie and Curtis 2006; Aledort *et al*, 2007; Jefferson *et al*, 2010) and one meta-analysis (Aiello *et al*, 2008) that included data on hand hygiene to reduce the spread of acute respiratory infections (ARIs) have been conducted. One review was specific to influenza (Aledort *et al*, 2007), but in general these papers relate to acute respiratory infections as a whole as there is little organism specific data. All reviews comment on the heterogeneity and often poor quality of studies done, but all conclude that hand hygiene can reduce episodes of respiratory illness. Two papers report pooled estimates of effect of 16 and 21% (Rabie and Curtis 2006; Aiello *et al*, 2008).

A systematic review of the evidence that face masks can prevent influenza transmission was undertaken by Cowling *et al* (Cowling *et al*, 2010b). It concluded that there is some evidence to support the use of either a SFM or respirator by an infected person to protect others but fewer data to endorse the wearing of a SFM to prevent the wearer from becoming

infected. However, it should be recognised that the evidence base is small and the quality of the studies reviewed was variable.

Using the schlieren optical method to visualise airflows around human subjects, Tang *et al* show that a cough projects a turbulent jet into surrounding air and that this can be blocked by wearing a respirator or redirected by wearing a SFM (Tang *et al*, 2009). More recently, Milton *et al* have shown that SFMs worn by influenza infected subjects can reduce the number of virus containing particles emitted. Larger virus containing particles ($\geq 5\mu\text{m}$) were reduced more than smaller particles ($< 5\mu\text{m}$); overall SFMs produced a fivefold reduction in viral aerosol shedding (Milton *et al*, 2010).

Prospective studies enrol participants (individuals, families, households) into randomised intervention trials and follow them over a period of time during which influenza activity is likely to be high. Studies have been performed in both community (homes, schools, university residences) and healthcare settings; in the latter the effectiveness of SFMs and respirators have been compared. There are indications that some of the interventions deployed in community studies may have had some benefit in certain situations though only one study has shown positive results with regard to primary intention-to-treat objectives (Talaat *et al*, 2011). Selected studies are described in more detail below. The difficulties and limitations faced by these intervention studies are outlined in Table 2.1.

- A study assessing the impact of a hand hygiene campaign on the incidence of laboratory confirmed influenza and absenteeism was conducted in Cairo, Egypt. The trial introduced an intensive hand hygiene programme to 30 schools over a 12 week period; 30 different

schools acted as controls. In the control arm there were 0.5 episodes per 100 student weeks of absence due to an influenza-like illness (ILI), in the intervention arm the rate was 0.3; risk reduction = 40% ($p < 0.0001$). The incidence of laboratory confirmed influenza (both A and B) between the control and intervention group was reduced by 50% ($p < 0.0001$) (Talaat *et al*, 2011).

- Aiello *et al* conducted a primary prevention study that recruited 1372 young adult residents in university accommodation. Volunteers were assigned to SFM use, SFM plus hand hygiene or a control arm for 6 weeks during an influenza season (06/07) in the US. Three hundred and sixty eight (32%) subjects reported symptoms of ILI and 94 samples were obtained for virological analysis; ten were positive for influenza (3.7%). Neither intervention resulted in a significant reduction in cumulative ILI incidence over the entire study period but during weeks 4-6 there was a significant reduction of 35% [95% confidence interval (CI), 9%–53%) to 51% (95%CI, 13%–73%) in ILI in the SFM plus hand hygiene group and during weeks 4 and 5 there was a significant reduction in ILI of 28% (95%CI, 2-47%) to 35% (95%CI, 2-57%) in the SFM only group (Aiello *et al*, 2010b). It is worth noting that the average use of a SFM each day was only 3.5 hours. While the authors suggest SFMs had the largest impact on transmission reduction, it is important to note that 'normal' hand washing continued to take place in all study arms; it was use of a specific hand sanitizer that was being assessed in the hand hygiene intervention arm.
- Cowling *et al* conducted a secondary prevention study. Index cases presented for medical care within 48 hours of symptom onset and

tested positive for influenza via a rapid antigen test. Members of the household of the index case (including the index case) were then randomised to interventions to reduce transmission. Interventions were i) control, ii) hand hygiene and iii) hand hygiene plus SFM. 259 households (794 individuals) were subsequently visited and samples were collected. The primary outcome was laboratory confirmed influenza in household contacts. Adherence to interventions varied and contamination between groups occurred. Less than half of the index patients in the SFM plus hand hygiene group reported regular use of a SFM during follow-up and adherence among household contacts was lower. Good adherence to the hand hygiene intervention was no better than 62% in any group. The SAR in the study was low (8%) and no differences were seen across the intervention arms. In a subgroup of households who implemented the interventions within 36 hours of symptom onset, transmission was significantly reduced (adjusted OR, 0.33 [95% CI, 0.13 to 0.87]) in the hand hygiene plus SFM group (Cowling *et al*, 2009a).

- MacIntyre *et al* also performed a secondary prevention study. Two hundred and eighty six adults from 143 households containing a child suffering a respiratory illness were recruited (influenza was detected in 21% of children). They were randomised to interventions that consisted of i) SFM, ii) respirator (FFP2/N95 mask, not fit tested) and iii) control. ILI was reported in 16%, 22% and 15% of adults in each group respectively; there were no statistically significant differences. Good compliance with mask use, defined as 'wore mask most or all of the time' over a 5 day period was reported by 21%. In a subgroup of adults who were adherent, use of either mask reduced their risk for ILI by between 60-80% (MacIntyre *et al*, 2009).

The majority of studies show some evidence of effect for the use of a face mask though it is difficult to say how this effect is mediated e.g. through reduced face touching or as a physical barrier to droplets. Furthermore, the beneficial effects of face masks are often seen in combination with hand hygiene interventions. Compliance with interventions has been problematic and another issue is the fact that the interventions in the secondary prevention studies are often only deployed after symptoms begin so missing periods of possible transmission when an index case is asymptomatic.

Two randomised studies have reported data on the use of face masks to reduce influenza transmission by studying nosocomial transmission between patients (naturally infected) and healthcare workers who attend them.

- The objective of Loeb's study was to compare SFMs with respirators (FFP2/N95) to protect healthcare workers from influenza (Loeb *et al*, 2009). Nurses working in Canadian emergency departments were randomised to a mask and asked to wear it whilst caring for patients with febrile respiratory illnesses during an influenza season. Four hundred and forty six nurses were recruited and the primary outcome was laboratory confirmed (PCR and/or serology) influenza. Influenza was diagnosed in 50 (23.6%) nurses in the SFM group and 48 (22.9%) nurses in the respirator group (absolute risk difference, -0.73% ; 95% CI, -8.8% to 7.3% ; $p=0.86$), indicating no significant difference between outcomes in the two arms. The vast majority of influenza diagnoses were made by serology; ILI was reported by only 11 nurses (nine in the SFM group and two in the respirator group, a non-significant difference) suggesting that the study was markedly under-powered for ILI and PCR based endpoints.

- MacIntyre and colleagues also compared SFMs with respirators (FFP2/N95) to protect health-care workers from acute respiratory infections (MacIntyre *et al*, 2011). Nurses and doctors (n=1922) working in emergency departments and respiratory wards were recruited from 24 hospitals in Beijing, China. A non-randomised comparator group was asked to continue with usual practice (n=481) while other recruits (n=1441) were cluster-randomised to one of three intervention arms: SFMs, fit-tested respirators, and non fit-tested respirators. Masks were worn during all working hours for four consecutive weeks. For each infection outcome, respirators (fit tested and non-fit tested) were associated with an approximate halving of risk compared with SFMs. However, after adjustment for clustering, the only significant finding was that non-fit tested respirators were more protective against clinical respiratory infection compared with SFMs (OR 0.48 [95% CI 0.24–0.98]; p=0.045). Overall event rates were low, in the randomised arms clinical respiratory infections were reported by fewer than 7% of HCWs and ILI by <1%. Laboratory testing confirmed influenza in <1% of HCWs. These low event rates compromise the power of the study. Interpreting this study as suggesting respirators have somewhat greater effectiveness than SFMs might indicate a more significant role for aerosol transmission.

The debate about whether respirators are needed routinely to protect HCWs from influenza continues. Until a trial is conducted that is large enough to capture a high event rate, a proportion of which are generated by aerosol mediated transmissions, we are unlikely to be able to conclude from randomised controlled trials (RCT) that respirators are superior for all situations.

Intervention studies designed to evaluate the effectiveness of specific items such as facemasks or respirators do not lend themselves to easy determination of the routes of transmission involved. While the studies discussed have the potential to give an indication of the 'real world' efficacy of interventions, they are unable to provide the emphatic evidence sought by governments and policy makers, especially with regards to modes of transmission. To date, the balance of evidence from randomised studies suggests that respirators seem no more effective than SFMs in preventing influenza transmission. Although this might suggest that the aerosol route of transmission is less significant than the droplet route, several other factors could have influenced study findings. Randomised studies of hand hygiene may be easier to interpret in relation to establishing the role of contact transmission, but not its relative importance compared with droplets and aerosols. A problem with using interventions to assess modes of transmission is that blocking one route still allows transmission to take place down other alternative (unblocked/open) routes. For example if contact transmission is blocked by hand hygiene, transmission could still occur via droplets and aerosols making the interpretation of any risk reduction complex (Briscoe 1984). Competing risk style models are required to make accurate inferences about the routes of transmission involved.

Table 2.1: Difficulties and limitations of community intervention studies.

<p>Although able to generate some data on the effectiveness of interventions most studies are unable to reveal which route(s) of transmission have been reduced; A respirator could reduce hand-to-face contact, droplet and aerosol exposure - which is most important?</p>
<p>The number of participants required and therefore the costs involved are considerable given the low clinical attack rates of influenza seen in recent seasons and the potentially modest effect size.</p>
<p>Use of clinical case definitions alone to identify patients with influenza is problematic (Call <i>et al</i>, 2005). Results from English GP-based sentinel virological surveillance in 2008/09 show that only 34% of samples taken from patients who present with an ILI are positive for influenza (McLean <i>et al</i>, 2009). In the US over recent years, the percentage of respiratory samples that test positive for influenza during an influenza season has been <20% (CDC 2011).</p>
<p>Studies based on a mixture of ARIs are able to generate more power, but have to assume that the contributions of different modes of transmission are the same for all respiratory viruses. Given the available data on influenza, RSV and rhinovirus transmission this is probably a false assumption (Goldmann 2000).</p>
<p>Most studies rely on PCR based identification of influenza from nose and throat specimens to assess outcomes. The ideal specimen is a nasopharyngeal sample (Sung <i>et al</i>, 2008; Spyridaki <i>et al</i>, 2009; Ngaosuwanukul <i>et al</i>, 2010) but this is often considered overly invasive in a community setting. Furthermore, viral shedding varies by day of illness so studies ideally need to sample early in disease and at multiple time points in both index cases and contacts.</p>
<p>It is important that interventions are initiated as early as possible when virus shedding is at its peak. This can be difficult to achieve when participants are recruited only after illness in an index case has begun.</p>
<p>A subject's compliance with study interventions e.g. face mask use and hand hygiene, is often low and this has proved to be a major obstacle. Compliance may be much higher in a pandemic because of perceived risk, but this is difficult to simulate for 'normal' seasonal influenza.</p>
<p>Confounding variables are difficult to eliminate in community infection studies. Although in theory randomised controlled trials eliminate confounding this is only the case if intention to treat analyses are used.</p>
<p>An intrinsic limitation of studies in healthcare settings is that the relative risk of transmission within the study context (the hospital) and outside (i.e. the household and community) is unknown; if most exposure to influenza occurs outside the healthcare context, such studies will always be limited in their ability to demonstrate a significant difference in intervention effectiveness even if one occurred.</p>

2.8 Human Challenge Studies

Experimental human challenge studies present an attractive way to study influenza transmission. Some of the earliest respiratory virus human challenge experiments took place during the 1918/19 influenza pandemic, when attempts were made to demonstrate the transmission of infection from symptomatic patients with presumed influenza to healthy volunteers (Rosenau 1919). These experiments were unsuccessful, probably because the volunteers were immune. The first successful influenza challenge study took place in 1936 when volunteers were infected with atomised suspensions of infected mouse lung (Smorodintseff 1937).

Notable findings from challenge studies in relation to transmission include the following;

- Henle published findings from over 200 volunteer exposures and identified the route of inoculation as important; infection by inhalation led to fever much more frequently than did nasal instillation (89% vs. 13%) (Henle et al, 1946).
- There is evidence to suggest that the infectious dose required for aerosol inoculation (0.6-3 TCID₅₀) is substantially lower than that required for intranasal inoculation (100-1000 TCID₅₀) (Alford et al, 1966; Couch et al, 1971; Douglas 1975; Knight 1980; Hayden et al, 1996). In Alford's study an H2N2 virus aerosol was produced using an atomiser which generated particles in the 1-3µm range. Twenty three volunteers (14 of whom had antibody titres to the challenge virus of ≤1:40) inhaled 10 litres of the aerosol which was delivered via a facemask. The dose of virus delivered ranged between 1-126 TCID₅₀; in the majority the dose was <5 TCID₅₀. Four volunteers developed clinical illness; virus was isolated from these and one other volunteer,

whilst seroconversion was seen in seven including all those who exhibited illness. Noting limitations of the study design and making an assumption that only 60% of the aerosol load inhaled will reach the LRT, the study reports that half of the volunteers with very low pre-existing antibody titres were infected with 0.3-6 TCID₅₀. In another study 30 TCID₅₀ of the same virus was given to volunteers via intranasal spray; 12 out of 30 (40%) became ill (Jao *et al*, 1965).

- In a study which attempted to compare natural and experimental influenza (induced by nasal inoculation), it was found that natural infections produced more fever, more cough and had a more marked effect on pulmonary function tests (Little *et al*, 1979). Possible explanations for this lie in differences between the infecting viruses themselves and the route of inoculation.
- Findings from studies that have assessed the use of the neuraminidase inhibitor (NAI) zanamivir seem to suggest that antiviral prophylaxis of the nose alone does not prevent natural influenza whereas orally inhaled zanamivir does (Calfee *et al*, 1999; Monto *et al*, 1999; Kaiser *et al*, 2000; Monto *et al*, 2002); this points to the pharynx and/or tracheobronchial tree as key sites for virus acquisition. In terms of routes of transmission, this data does not allow us to discriminate between droplets and aerosols as both can reach the pharynx, but it does suggest that the contact route may not play a dominant role.

In analysing the findings from experimental challenge studies it should be recognised that that the inoculation methods employed are unlikely to accurately replicate transmission that occurs in natural settings. For example the size, concentration and viral load of aerosols and delivery methods that have been used to achieve infection are rather artificial.

2.9 Animal Studies

Animal studies have played an important role in advancing our knowledge about influenza and its management. Indeed, it was through the use of a ferret model of infection that influenza was first isolated. Studies showed that throat washings obtained from humans, who had an influenza-like illness, could be used to infect ferrets and produce a very similar disease (Smith *et al*, 1933). Studies seeking to improve our understanding of influenza transmission have often employed mice or ferrets. However, the murine model has fallen out of favour because researchers have experienced difficulties in getting the virus to transmit consistently (Schulman and Kilbourne 1963; Lowen *et al*, 2006) and the guinea pig has been proposed as an alternative (Lowen *et al*, 2006). Using these animals, transmission and factors related to the host, the environment and the virus itself have been explored.

The droplet and aerosol routes of transmission dominate in transmission experiments with animals. Unfortunately it is not possible to discriminate between them in most models (Andrewes and Glover 1941; Schulman 1968; Lowen *et al*, 2006; Mubareka *et al*, 2009) though it has been argued that the experimental methods described favour the operation of aerosol over droplet transmission (Tellier 2009). We should caution against dismissing the contact route as minor; experimental methodologies may bias against it and the markedly different social and physical behaviours of humans compared to small mammals are probably critical.

There seems little doubt that some environmental factors e.g. temperature and humidity can affect transmission (Lowen *et al*, 2007; Lowen *et al*,

2008; Lowen and Palese 2009). Experiments with guinea pigs show that aerosol/droplet transmission is reduced at high temperatures and RH whilst contact transmission is preserved. These findings led the authors to suggest that contact transmission predominates in tropical climates and may explain the lack of a well defined influenza season (Lowen *et al*, 2008). However, the extent to which all these findings can be generalised to human transmission is uncertain and scientifically challengeable.

Through use of animal models a better understanding of the viral determinants of transmission is developing, though the variety and interplay of traits is complex, some seeming to hinder transmission whilst others permit it through different routes. It is likely that viral properties (e.g. fitness for replication, receptor preferences) help determine infectiousness and modes of spread (Nicholls *et al*, 2008; Belser *et al*, 2010).

Despite the development of valid and reliable animal models it requires a leap of faith to extrapolate animal findings to humans when considering influenza transmission. Disease pathogenesis including immunopathology will differ and host factors that contribute to transmission can vary between animal models, for example, symptoms and social and physical behaviours. In humans the existence of super-spreaders appears likely and the possibility that different social behaviours and interactions can affect transmission seems logical. It is difficult to study such human phenomena in animals. Furthermore, animal models do not allow us to test NPIs to reduce transmission. So whilst animal models are generating useful and important findings their application to humans will always be debatable.

2.10 Modelling Influenza Transmission

Modelling is an attempt to predict an outcome based on variables (either known or hypothetical) associated with the outcome. Using modelling, a number of authors have tried to estimate the importance of the various routes of influenza transmission with infection resulting from a particular route being the outcome. The development of a plausible model, however, is not straight forward because a large number of parameters need to be taken into account. Furthermore, whilst some of the parameters have been well characterised many others have not and this undermines the reliability of a model.

A number of modelling scenarios have been constructed that combine defined physical dynamics with biologic processes to estimate outcomes. Whilst most support the concept that all transmission routes can be important given the right circumstances, there does appear to be some divergence between those who conclude that droplet transmission is significant (Teunis *et al*, 2011) and those who conclude it is less significant (Atkinson and Wein 2008; Nicas and Jones 2009; Spicknall *et al*, 2010). Despite droplet particles being high in number and having high infectivity potential, it is likely that their inability to reach target cells and data which reveals that the infectious dose in the URT is higher than the LRT (Tellier 2006) are responsible for the modelling findings against droplets. Some models suggest a significant role for contact transmission (Nicas and Best 2008; Nicas and Jones 2009; Spicknall *et al*, 2010) though model outputs are highly dependent on estimates of infectious dose.

There are however significant limitations to each of these models;

- The empiric data that they rely on is weak. Many crucial variables arise from studies undertaken many years ago and both the reliability and validity of data is questionable.
- The assumptions and data that some models have used is open to debate (Tellier 2009).
- The models are restricted to certain scenarios, e.g. a coughing patient being visited in a bedroom. They cannot possibly take account of the huge variety of other factors, e.g. patients being mobile rather than bed-ridden, particle emission through talking, breathing and sneezing as opposed to coughing alone, heterogeneity in particle emission (e.g. super-spreaders) and room ventilation changes through door and window opening.

Despite drawbacks, modelling has highlighted several important determinants of infection risk, for example viral shedding, infectivity of influenza at different sites, host density and viral transfer efficiencies (Jones 2011). By focusing future research on these areas and obtaining better data, models can be improved and they will become invaluable in helping us to appreciate the roles played by the different routes of transmission.

2.11 Summary

The evidence base for influenza transmission is largely derived from studies that have assessed; virus deposition and survival in the environment; the epidemiology of disease in hospitals, nursing homes and other closed or semi-closed settings; prospective pharmaceutical and non-pharmaceutical interventions (NPIs) in the setting of natural infection;

animal models; and mathematical modelling of transmission. Whilst most studies contribute in some way to the evidence base, very few are conclusive; some (especially retrospective observations) are weakened by confounding factors and others (animal and laboratory studies) can be difficult to interpret in the context of 'normal' human interactions. Furthermore, investigating routes of transmission was seldom the primary aim of the studies reviewed.

Studying influenza transmission is difficult; seasonality, unpredictable attack rates, role of environmental parameters such as temperature and humidity, numbers of participants required and confounding variables all present considerable obstacles. A range of studies done to date have failed to provide definitive answers. Furthermore, they have revealed little about the routes of transmission and the relative contributions made by each. For a table summary of the evidence for each route of transmission see Appendix 2.7.

Further research into routes of transmission is needed and has been called for by many authorities (Bell 2006a; ECDC 2007; IOM 2007; Aiello *et al*, 2010a). A recent discussion paper following a series of studies funded by CDC, recognised ongoing evidence gaps to be "the relative contributions of influenza virus transmission modalities to disease spread" and "the efficacy of different types of masks, hand hygiene, and combinations of personal protective measures for reducing transmission of influenza" (Vukotich *et al*, 2010).

2.12 The Scope of this Thesis

Research and study towards this thesis was undertaken on the background of the evidence base presented and an appreciation of the principal scientific and public health policy questions that exist with regard to influenza transmission. Two main lines of investigation were undertaken;

1. The consideration, design and conduct of a clinical trial to collect data on nasal shedding and environmental deposition of influenza virus:

The proposed routes of transmission all appear plausible. The evidence base suggests that influenza virus can remain viable on surfaces and hands for periods which are consistent with onwards transmission and there is good evidence that humans infected with influenza produce respiratory droplets and aerosols which contain influenza virus and are therefore of infectious potential. However, there is a relative lack of data from field settings involving naturally infected patients in support of these findings. The detection of virus in the environment (on fomites and in air) is the first step to demonstrate that transmission occurs via such routes, but data to confirm the presence and amount of infectious virus around patients and that infection can be transmitted specifically by fomites and /or air is sparse. The opportunity to collect such data arose during the 2009 influenza pandemic and the resulting study is presented in Chapter 4.

2. The consideration, design and conduct of a novel method to study influenza transmission in humans:

An influenza transmission strategy development group was convened and met to discuss, develop and agree on suitable designs for pragmatic research studies. The group considered the lack of

understanding about the modes of transmission, specifically in humans, critical to address in order to enable further research and policy development. Noting the advantages and disadvantages of a number of study methods, the group considered influenza challenge studies (involving the deliberate infection of volunteers) to present an alternative way of studying infection. The development of a human challenge model to study transmission is presented in Chapter 5.

In addition, work was undertaken to validate air sampling methodologies used in the work above (Chapter 3).

Chapter 3:
Experiments to Investigate
Techniques for the
Sampling and Detection of
Airborne Influenza Virus

3.1 Abstract

Background

Indirect evidence exists to support the concept that influenza virus can be transmitted between humans via small aerosolised particles (droplet nuclei). The significance of the aerosol route of transmission is unknown and methodological challenges to virus sampling and detection in air limit our insights. A bioaerosol sampler has been developed which is able to size fractionate collected airborne particles and influenza has been detected in such particles. Prior to use of the sampler in UK studies, a two stage validation experiment was conducted.

Methods

Two laboratory based experiments were performed that involved aerosolisation of influenza with subsequent attempts at both virus collection and detection (by PCR and culture). In the first experiment sampling took place following release of virus at close range to the sampler over a short time period with and without viral transport medium. Attempts to simulate more natural conditions were made in the second experiment with longer sampling times following virus aerosolisation into a room.

Results

Influenza could be detected by both PCR and culture techniques in both experiments following the collection of air particles by the sampler. In the second experiment, detection was possible after 1-3 hours sampling time, when the samplers were placed at distances of 3 and 7ft from the virus source and whether or not viral transport medium was used.

Conclusions

We have been able to validate use of the samplers for planned field studies despite some limitations and inconsistencies seen in the results. Importantly virus has been detected in particles that are small enough to be respired. If present in the environment around susceptible individuals such particles could mediate the aerosol transmission of influenza.

3.2 Introduction

Any microorganism can become airborne when associated with particles that are dispersed in air (aerosols). The propensity to stay airborne depends on the aerodynamic sizes of particles; the larger the particle, the quicker it will fall to the ground. For a 3m fall, particles $>100\mu\text{m}$ in diameter will settle in seconds, particles $>10\mu\text{m}$ will settle in minutes whilst particles less than $<3\mu\text{m}$ in diameter can remain suspended in air for several hours (Nicas *et al*, 2005). It should be appreciated that whilst small particles make up the vast majority of particles contained in a respiratory spray (particles emitted by individuals during expulsion events e.g. coughing and sneezing), larger particles ($>10\mu\text{m}$) make up the vast majority of the volume and therefore carry the majority of microorganism load.

Particles laden with microorganisms can gain entry to the human respiratory tract by the actions of inhalation and respiration. Inhalation can bring particles into contact with the upper respiratory tract but only particles $\leq 10\mu\text{m}$ can reach the tracheobronchial tree and only particles $<5\mu\text{m}$ can be respired and reach the alveolar region (Soderholm 1989).

For influenza to be transmitted via aerosols it must a) be released from an infected person in a bioaerosol, b) survive in the aerosol and c) reach target cells in sufficient concentration to initiate infection (i.e. be present as an infectious dose). The ability to survive has been confirmed in laboratories (see Chapter 2, page 42), but to support the transmissibility of influenza via aerosols we would expect to be able to detect infectious virus from aerosols released into the environment by humans. Despite the advancement of molecular techniques over recent decades (e.g. PCR), it

remains that the detection of live virus from natural bioaerosols has not been shown. The detection of influenza in aerosols presents a number of challenges;

- Influenza is sensitive to dehydration and ultraviolet (UV) radiation.
- Sufficient virus needs to be collected to enable culture. This is challenging because concentrations in air are often low and they are rapidly diluted in air as distance from the source increases.
- Virus capture often requires the use of filters which complicate handling and recovery.
- PCR allows great precision in identifying virus but it does not tell us whether the recovered virus is viable (and therefore infectious).

Many types of aerosol samplers and analytical methods have been used over the years and the evolution of these instruments and methods is contributing to progress in this field. In general samplers rely on the aerodynamic size of particles and the properties they exhibit in relation to adhesion, Brownian motion, thermal gradients and inertia. Viruses can be collected on solid surfaces, filters or in liquid. The efficiency of a sampler is an important measurement and can constitute capture efficiency and/or the efficiency of viral recovery (the latter involving virological detection methods) (Verreault *et al*, 2008).

Contemporary efforts to detect influenza virus in aerosols have been successfully achieved by a number of groups, both in the laboratory (Hogan *et al*, 2005; Blachere *et al*, 2007; Pyankov *et al*, 2007; Fabian *et al*, 2009a) and from around patients (Fabian *et al*, 2008; Blachere *et al*, 2009).

- Fabian *et al* have published work on the ability of four aerosol samplers to capture aerosolised virus (Fabian *et al*, 2009a). They used both

molecular (PCR) and infectivity assays to detect virus. The samplers were; 1) a liquid impinger that could accommodate liquid collection media, 2) a cassette with Teflon filter, 3) a cassette with a gelatine filter and 4) a compact cascade impactor. All samplers collected virus detectable by PCR but the liquid impinger recovered live virus more effectively than the other samplers. The authors put this down largely to the effect of VTM assisting virus survival. They have also demonstrated that optimisation of molecular biology methods to improve virus detection is important (Fabian *et al*, 2009b).

- Scientists at the National Institute of Occupational Safety and Hygiene (NIOSH), US, designed and developed a cyclone-based bioaerosol sampler (Lindsley *et al*, 2006). Following aerosolisation of influenza virus they were able to successfully collect and detect virus (by PCR). Furthermore, collected particles were size fractionated allowing particles of a respirable size to be identified (Blachere *et al*, 2007). They went on to use the samplers in medical care facilities and demonstrated that collected air particles contained influenza (Blachere *et al*, 2009).

To contribute to the evidence base concerning the potential for influenza to be transmitted via aerosols, studies involving the sampling of air around infected subjects were planned. To this end a number of air sampling units and accessory equipment were loaned by collaborators at NIOSH. To prepare for the use of the samplers in a field setting, two laboratory studies were undertaken and a number of variables were tested.

We hypothesised that;

- a) Increasing lengths of sampling time will result in more virus being detected by PCR, but that sampling over a shorter period will result in

more infectious virus being detected by culture (as virus survival declines with time).

- b) Sampling further away from the source will result in less virus being detected (by both PCR and culture).
- c) Use of VTM may help preserve viable virus.

Investigations were carried out in April (Experiment 1) and September 2009 (Experiment 2) at facilities of The Health and Safety Laboratory (HSL), Buxton, UK.

3.3 Experiment 1

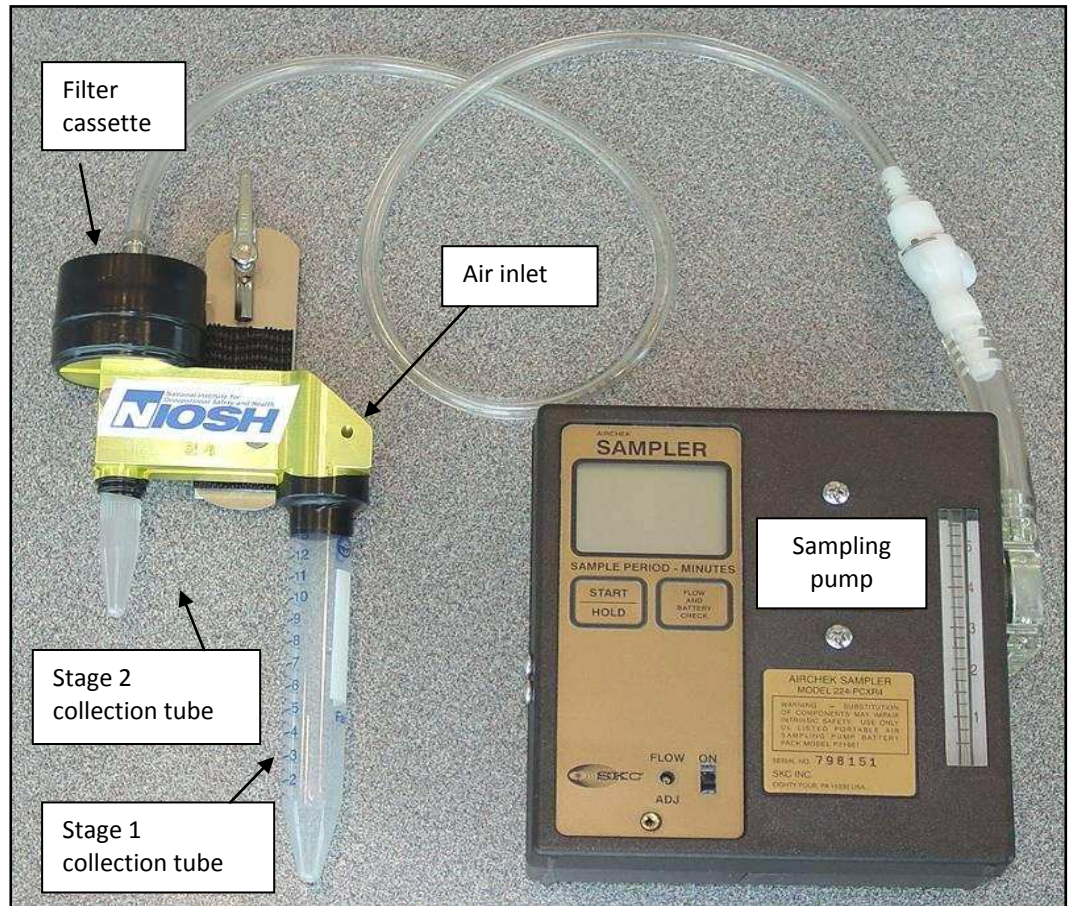
Objective: To capture aerosolised influenza virus released into a laboratory safety cabinet using a bioaerosol sampler and to detect virus by PCR and culture techniques.

3.3.1 Equipment and Methods

Aerosol samplers:

A two-stage cyclone aerosol sampler was used to collect aerosol samples. The sampler draws in air and collects and size fractionates (via two collection stages and a filter) airborne particles. At a flow rate of 3.5 l/min, the first stage collects particles with a diameter $>4 \mu\text{m}$, the second stage collects particles with a diameter of $1\text{--}4 \mu\text{m}$, and the filter collects particles with a diameter $<1 \mu\text{m}$ (Figures 3.1 and 3.2). The upper particle size limit of detection is not definitively known but is likely to be around $100 \mu\text{m}$ based on the fact that the collection efficiency of $80\mu\text{m}$ particles is approximately 20%. The sampler conforms to the American Conference of Governmental Industrial Hygienists/International Organization for Standardization criteria for respirable particle sampling.

Figure 3.1: NIOSH BC 251 two-stage cyclone aerosol sampler shown connected to a sampling pump.

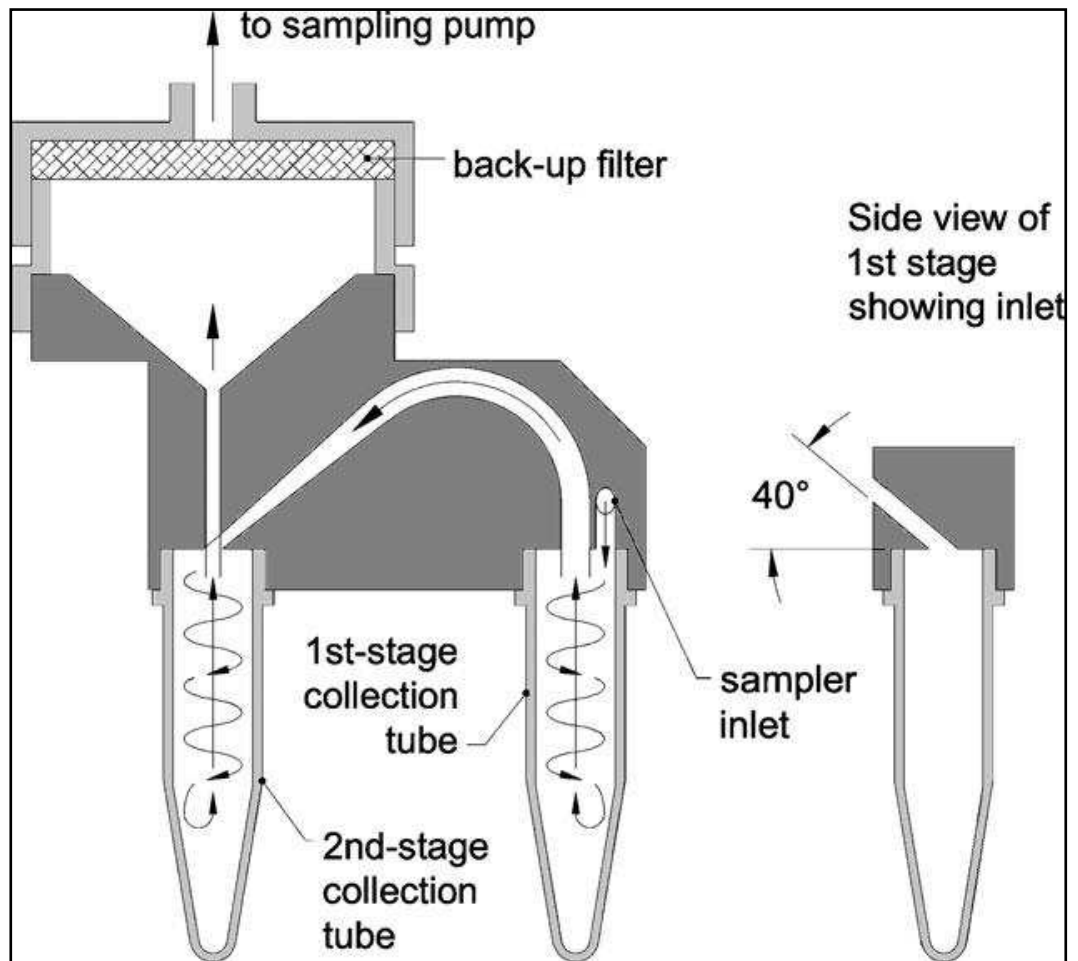


Footnote: Air is drawn into the sampler through the inlet on the sampler body above the 15mL tube, and exits through the black filter cassette on the top of the sampler.

The experiment was run on two occasions:

1. Using VTM - In an attempt to aid virus survival, the stage 1 tube (15mL) was charged with 750 μ l VTM (EMEM supplemented with 0.125% BSA, 25mM HEPES, Penicillin/Streptomycin and non-essential amino acids) and the stage 2 tube (1.5mL) was charged with 250 μ l.
2. Without VTM.

Figure 3.2: Diagram of the cyclone two-stage aerosol sampler.



Footnote: Arrows show the path of the air as it flows through the sampler. Figure courtesy of William Lindsley.

A midget impinger air sampler (SKC Ltd, US) was also assembled for parallel tests. Impingers are Pyrex glass bubble tubes designed to collect airborne material into a liquid medium. Ordinarily, midget impingers collect air particles by bubbling air through a large volume of liquid. In this study however, it was likely that the concentration of influenza virus recovered would be small and a large volume of liquid would dilute this further. In addition, vigorous bubbling of the fluid may render some of the recovered virus particles non-viable (Agranovski *et al*, 2004). A smaller volume fluid (750µl) was therefore chosen. The impinger was connected to

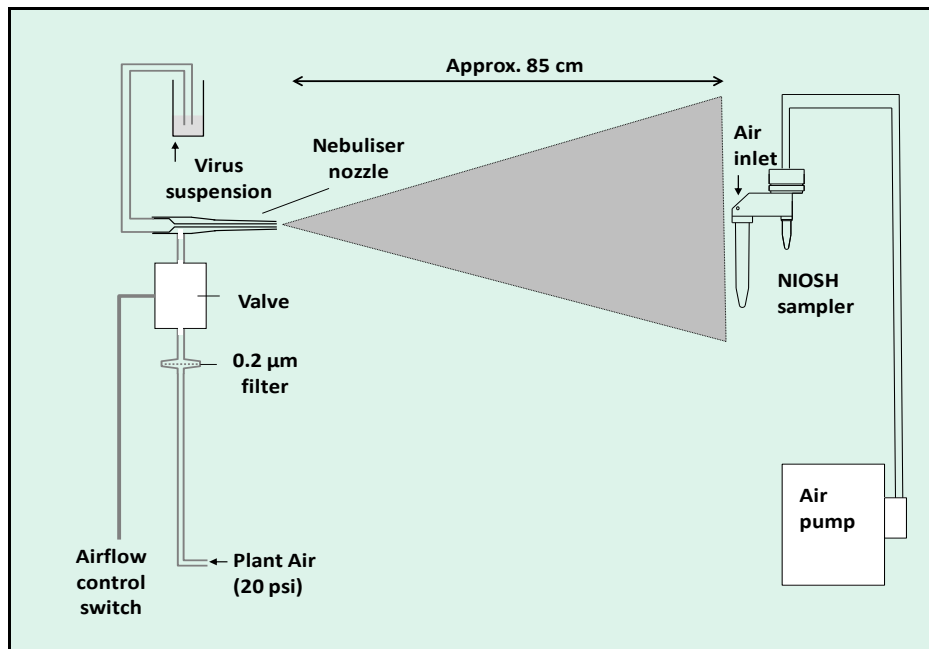
a vacuum pump at flow rate of 1L/min. A HEPA filter cartridge was placed between the impinger and sampling pump to prevent contamination of the sampling pump.

The samplers were positioned within a 0.42m³ (120 x 50 x 70cm) Class II Microbiological Safety Cabinet immediately opposite a pulsed compressed air atomiser (Figure 3.3). The atomiser generates a poly-dispersed aerosol covering a size range <1 to >200µm with approximately 50% of the particles being <20µm and 10% being >100µm. This compares well with the particle size distribution of a cough (*Nicas et al, 2005*). The sampling distance from the atomiser to the samplers was approximately 85cm.

Virus:

A variant of influenza virus A/H1N1/PR/8/34 [adapted for growth on Madin-Darby Canine Kidney (MDCK) cells], obtained from the American Type Culture Collection (LGC Promochem Ltd, UK) was used for this experiment. MDCK cells were obtained from the European Collection of Cell Cultures and cultured as directed by the supplier. High titre stocks of virus were grown on cultured MDCK cells as previously described (Gauth and Smith 1968). Virus was harvested when a cytopathic effect was visible on 80-100% of the cell monolayer. Cellular debris was removed from the crude virus preparation by centrifugation at 1000xg. This clarified viral preparation was subsequently stored at -80°C. Prior to use in aerosol sampling studies, influenza virus was concentrated by ultracentrifugation at 100,000xg for 2.5 hours at 4°C. The supernatant was aspirated and the viral pellet re-suspended in PBS containing 0.2% (w/v) Fraction V BSA overnight at 4°C. Virus collected from approximately 36mL of crude preparation was re-suspended in 6mL PBS + 0.2% BSA. The concentration of virus suspension was between 10¹² and 10¹³ PFU/mL.

Figure 3.3: Diagram depicting the experimental set up.



Footnote: Aerosolised particles (represented by the grey area) are produced from a nebuliser and sampled by the NIOSH sampler.

The atomiser was charged with 5mL of the virus test suspension. Vacuum pumps were switched on and a simulated cough was achieved with a 1 second pulsed spray of the influenza test suspension. A 1 second pulse releases approximately 460µl of suspension; this should contain approximately 2.5×10^{12} PFU. Air was sampled for a period of 5 minutes before the vacuum pump was switched off. The cabinet airflow was switched back on to remove residual bioaerosol particles and to permit safe handling of the samples. Liquid samples were removed from the samplers. The stage 2 tube was reconstituted to 750µl VTM. The filters were placed in a 6-well tissue culture dish and soaked in 750µl virus transport medium for 4 hours. Liquid samples were then stored at -80°C before processing.

Virus detection:

The titre of influenza virus present in the samples was determined by plaque dilution assay (Gaush and Smith 1968; Gray 1999). Plaques were counted on day 3 post-infection, and the titre in plaque-forming units per mL of influenza virus in the original sample calculated. The presence of virus was also determined by PCR. RNA was extracted from 200µl of each sample using a Mag-Max Viral RNA isolation kit (Ambion). Detection of a conserved region of the matrix gene of influenza A and the XenoRNA-01 internal control was performed using the AIV-M Primer Probe mix (Applied Biosystems) according to the manufacturer's instructions on a BioRAD i-Cycler. A control RNA (10,000 copies/mL) was serially diluted and used to establish a quantification curve. This curve allowed the cycle threshold (Ct) values of all the other curves to be matched against it and the amount of viral RNA present to be quantified.

3.3.2 Results

The first run of the experiment employed a NIOSH and an SKC sampler, both using VTM. Influenza virus was detected by PCR and Plaque Assay (PA) from both samplers. All particle sizes collected by the NIOSH sampler were positive by PA (i.e. detected live virus). Only the filter of the NIOSH sampler detected virus by PCR. In the second run, no VTM was used. Again, PCR and PA detected influenza virus from both samplers. All particle sizes collected by the NIOSH sampler demonstrated viable virus by PA whilst stage 2 and the filter demonstrated virus by PCR (Table 3.1).

Table 3.1: Results from Experiment 1 (runs 1 and 2).

Run 1: Virus transport medium used in stage 1 and 2 collectors of the NIOSH samplers		
Sampler (particle size collected)	Plaque Assay (PFU/mL)	RT-PCR (Copies/mL)
SKC Impinger (*)	4.7×10^4	9.4×10^4
NIOSH Sampler stage 1 (>4 μ m)	1.72×10^2	0
NIOSH Sampler stage 2 (1-4 μ m)	2.4×10^3	0
NIOSH Sampler filter (<1 μ m)	2.32×10^2	3.2×10^5
Run 2: No Virus transport medium used in stage 1 and 2 collectors of the NIOSH samplers		
Sampler (particle size collected)	Plaque Assay (PFU/mL)	RT-PCR (Copies/mL)
SKC Impinger (*)	2.9×10^4	5.16×10^7
NIOSH Sampler stage 1 (>4 μ m)	4.4×10^3	0
NIOSH Sampler stage 2 (1-4 μ m)	3.4×10^4	6.86×10^5
NIOSH Sampler filter (<1 μ m)	1.76×10^2	2.02×10^4

*Footnote: *The impinger will collect a wide range of particles but the upper and lower size ranges are not known. Stage 1 of the NIOSH sampler captures particles <1 μ m, stage 2 captures particles between 1 and 4 μ m and the filter captures particles >4 μ m.*

3.4 Experiment 2

Objective: To capture aerosolised influenza virus released into a controlled air chamber (CAC) using a bioaerosol sampler and to detect virus by PCR and culture techniques.

3.4.1 Equipment and Methods

Except where stated below, the materials and methods used were identical to those outlined in Experiment 1. The CAC is of a size comparable to a hospital side room or bedroom in a house (3m x 3m x 4m = 36m³). This approximates field conditions and allows the released aerosol to disperse in a more realistic fashion compared to the small volume within the microbiological safety cabinet used in Experiment 1. It also allowed samples to be taken from a variety of locations. The samplers were positioned at a height of 150cm from the floor (a similar height to that of a patient's head when sitting in bed) and at distances of either 3 or 7ft from a pulsed compressed air atomiser. The temperature within the CAC was 23°C and RH was 29%. The atomiser was charged with 5mL of virus test suspension. The CAC access door and portholes were closed and sealed, and the airflow was switched off. It is known that approximately 10% of air will be lost from the CAC system via the extractor fan. The sampling pumps were switched on and a simulated cough was achieved with a 1 second pulsed spray of influenza virus test suspension. This was repeated once every hour over a 3 hour period (time points 0, 1 and 2 hours). Six NIOSH samplers were used and they varied with respect to; i) amount of VTM used in the first and second stage collection tubes, ii) distance from the aerosol source and iii) sampling time (see Table 3). A midjet impinger (SKC) was again used as a 'control' and ran for 3 hours at 3ft from virus source.

On completion of sampling, the chamber air was purged to remove any residual bioaerosol particles to permit safe handling of the samples. Liquid samples were removed from the samplers. As significant evaporation of VTM had occurred the stage 2 tubes from samplers 1-5 were reconstituted to 750µl using VTM. Stage tubes 1 and 2 from Sampler 6 were washed with 750µl of VTM. The filters were placed in a 6-well tissue culture dish

and soaked in 750µl virus transport medium for 4 hours. Liquid samples were stored at -80°C before processing.

3.4.2 Results

All the samplers collected and detected influenza virus by either PCR and/or PA. There does not appear to be any qualitative or quantitative relationship between the PCR and PA results. No formal statistical analysis were performed due to the small dataset.

PCR

- Of the 18 samples collected by the NIOSH samplers, virus could be detected in all but two.
- Longer sampling time appeared to lead to the detection of more virus (sampler 3 > 2 > 1) with the exception of the filter from sampler 1.
- Sampler 4 which was positioned at 7ft collected less virus than sampler 3 positioned at 3ft.
- Sampler 5 had an increased volume of VTM and collected less virus than its counterpart (sampler 3) which had a 'standard' volume of VTM.
- The sampler not using VTM (sampler 6) collected more virus than the sampler using VTM (sampler 3) in stage 1.

Plaque Assay

- Of the 18 samples collected, 12 contained live virus.
- On 4 out of 6 occasions, samples from the filter were negative.
- Sampling over a shorter time period appeared to lead to the recovery of more live virus (sampler 1 > 2 > 3).
- Sampling at a greater distance from the source found more live virus (sampler 4 vs. 3).

- Sampler 6 with no VTM collected more live virus in each size fraction than sampler 3. Sampler 5 which had the highest volume of VTM failed to demonstrate any live virus.

Table 3.2: Results from Experiment 2.

Sampler	Sampling Time (Hrs)	Distance from source (ft)	Stage	Volume of VTM	RT-PCR (copies /mL)	Plaque Assay (PFU/mL)
1	1	3	1	750	19	9
			2	250	15	9
			Filter	N/A	38300	0
2	2	3	1	750	134	9
			2	250	411	3
			Filter	N/A	19	0
3	3	3	1	750	27170	3
			2	250	12170	3
			Filter	N/A	653	0
4	3	7	1	750	69	6
			2	250	1121	6
			Filter	N/A	0	12
5	3	3	1	1500	2441	0
			2	500	13	0
			Filter	N/A	414	0
6	3	3	1	0	143200	12
			2	0	0	6
			Filter	N/A	83	6
SKC	3	3	-	750	67460	0

Footnote: Stage 1 captures particles <1 μ m, stage 2 captures particles between 1 and 4 μ m and the filter captures particles >4 μ m. VTM = Viral transport medium

3.5 Discussion

We were able to demonstrate that the NIOSH bioaerosol sampler can collect influenza virus in all three particle size ranges following virus release into air. Virus was detected by both PCR and culture techniques. The fact that virus was detected by culture on particles that are small enough to be respired at a distance of 7ft from the source is in itself important. If this finding were to be confirmed in a field setting around an infected patient it would add to the evidence that the aerosol route of transmission is at least possible.

Experiment 1 was designed to assess whether the use of liquid medium could successfully be used as part of the sampling system, as it is theoretically attractive to use VTM to help preserve the virus (Agranovski *et al*, 2004; Fabian *et al*, 2009a). Both runs of the experiment were successful suggesting that VTM can be employed though its use may not be obligatory. It should be recognised that the sampling time was short. The NIOSH sampler was roughly comparable to the SKC sampler.

Experiment 2 was an attempt to simulate natural conditions by releasing virus into a volume of air that might commonly exist around an infected patient. Releasing virus into a large volume of air makes detection more difficult due to dilution and drying effects. Furthermore, the ability to detect aerosolised virus by PCR in these circumstances is likely to be much more sensitive compared to the detection of live virus by PA; PA requires >1 viable infectious unit per inoculum to be present whereas PCR can detect small amounts of viral RNA, irrespective of viability.

A lack of data due to a limited number of experiments and a wide variation in results obtained from quantitative tests hampers our ability to rigorously test our hypotheses. Formal statistical analysis cannot be performed but we may speculate that;

- Culture of virus collected via the filter is difficult; particles collected here are the smallest and perhaps most vulnerable to dehydration, especially when in association with a porous material. In addition, the filters tend to carry a static charge, which might affect the viruses.
- Sampling over a short time period is beneficial for culture (compare results from samplers 1, 2 and 3). Increasing lengths of sampling time resulted in more virus being detected by PCR for stages 1 and 2.
- The findings relating to sampling over different distances are less clear. Samplers closest to source collected more virus by PCR but not by culture. A continuous flow of air through the sampling apparatus may reduce virus survival within the sampler compared to room air and captured virus at 3ft may spend more time in the sampler than virus captured at 7ft.
- The benefit of VTM is not clear; no other sampler collected more live virus than sampler 6, which did not use VTM. In contrast the sampler using increased volumes of VTM found no live virus; this could be a result of i) dilution of any virus that was collected below the level of detection for plaque assay ii) an effect on the efficiency of particle sampling by the sampler itself.

There are other limitations to this work. Firstly it is not clear why some samples failed to yield a positive PCR result, whilst the same sample contained detectable virus in the PA. Due to time constraints the PCR method was not fully optimised; improved sensitivity could likely have been achieved had this been done. Secondly, the mechanisms and the

amount of virus that an infected individual releases will be much more complex than can be achieved in an experimental set up; an arbitrary value of one 'cough' per hour was chosen in this experiment. Furthermore, we recognise that conditions such as air flow, temperature and humidity which were controlled in our experiment will vary both during and between different settings. In a hospital room for example, the presence of air conditioning, an open window or a door opening and closing will affect the concentration of bioaerosols in the air. This in turn will affect the ability to collect and detect them. Thirdly, no attempt has been made to analyse how the addition of a volume of VTM in each of the collection tubes affects the performance of the sampler. It could impact on the dynamics of airflow through the sampler with an effect on the sizes of particles trapped by each stage. Finally, an assessment of the filters sampling efficiency could not be made. Whilst the total amount of virus released could be estimated, the amount associated with specific particle sizes is unknown. Furthermore, we know that the vast majority of virus will be associated with the largest particles but the sampler's ability to collect these large particles is significantly impaired.

3.6 Conclusion

In the experiments described, the NIOSH aerosol sampler is able to collect particles containing influenza virus and this was detected using both PCR and culture techniques, the latter demonstrating the capture of live virus. Particles of sizes >4 , 1-4 and $<1\mu\text{m}$ were all shown to carry virus and virus could be detected over a variety of sampling times and distances from its source. The use of VTM within the collection tubes does not appear to enhance the detection of live virus. Despite limitations, these experiments do support the utility of the equipment and methods used to collect airborne influenza virus.

3.7 Acknowledgements

I would like to thank the microbiology team at HSL (Catherine Makison, Jonathan Gawn, Claire Bailey and Brian Crook) who collaborated in this work.

Chapter 4:
Shedding and
Environmental Deposition
of Influenza Virus

4.1 Abstract

Background

Influenza transmission is an area that is poorly understood and hotly debated. A better appreciation about the extent to which virus is deposited by infected individuals into the environment and whether deposited virus has the ability to infect new hosts, i.e. whether it remains viable, is important to our understanding of the routes and mechanisms of transmission. This study was conducted to collect data on subjects who were infected with influenza. The primary objectives were to correlate the amount of virus detected in a patient's nose with that recovered from his/her immediate environment (on surfaces and in the air), and with symptom duration and severity.

Methods

Adults and children, both in hospital and from the community, who had symptoms of influenza infection were enrolled and followed up.

Information about symptoms was collected and samples were taken including nose swabs, swabs from surfaces (e.g. door handles, remote controls) and air samples from around patients. Samples were tested for the presence of influenza virus, using PCR to detect virus genome and culture to detect viable (live) virus.

Results

42 subjects with confirmed infection were followed up. Mean duration of virus shedding was 6.2 days by PCR and 4.2 days by culture. Over 25% of cases remained potentially infectious for at least 5 days. From surface swabs collected in the vicinity of 40 subjects, 15 (38%) subject locations were contaminated with virus. Overall 35 of 662 (5.3%) surface swabs

taken were positive for influenza, two (0.3%) yielded viable virus. Subjects yielding positive surface samples had significantly higher nasal viral loads on illness Day 3 and more prominent respiratory symptom scores. Room air was sampled in the vicinity of 12 subjects and PCR positive samples were obtained from five (42%). Particles small enough to reach the distal lung ($\leq 4\mu\text{m}$) were found to contain virus, though we were unable to detect the presence of live virus.

Conclusions

Despite some limitations caused by the small number of subjects recruited, important observations have been made. The duration of viral shedding is in line with other published studies. The data on viral deposition suggests that either swabbing and/or laboratory methods are insensitive or that virus deposited by infected patients does not contaminate the vast majority of surfaces in high titre. It is likely that both explanations contribute. Contact transmission of influenza via surfaces may be less important than current infection control policies and public guidance documents imply. Findings from the air sampling component of the study show for the first time that influenza can be detected in the air around identifiable influenza patients and add to the accumulating evidence in support of the potential for aerosol transmission of influenza.

4.2 Introduction

As pandemic mitigation strategies have been developed over recent years it has become very clear that influenza transmission is one area that is poorly understood and hotly debated. Distinguishing the relative importance of the various modes of transmission is critical for the development of infection control precautions in healthcare settings and in the home.

An important and to date relatively neglected area of research concerns the presence of virus in the environment, virus potentially available for transfer to new hosts. Through the release of respiratory sprays (generated by actions such as coughing, sneezing and talking), virus may be deposited on hands, surfaces and into the air. For these viruses to cause infection in new hosts, a number of prerequisites exist;

1. Virus must survive in the environment.
2. Virus must reach target cells in a new host.
3. Enough virus must reach target cells such that an infectious dose is achieved and infection initiated.

A number of laboratory studies have confirmed the ability of influenza to survive in the environment (see Chapter 2, pages 38 & 42) but surprisingly few studies have attempted to investigate the presence, quantity and viability of virus in the natural environment around infected patients. Laboratory studies are important but they cannot adequately replicate the spatial layout, content and conditions of field settings. Furthermore they cannot take account of human behaviour and interactions. There is therefore a need to obtain data concerning

deposition of virus in natural settings in order to improve our understanding of the routes and mechanisms of transmission.

In all previous research on influenza virus excretion, viral shedding has been determined by measurement of the quantity of virus recoverable from the patient's nasopharynx, i.e. virus has been recovered by a deliberately performed invasive technique. These so called 'viral shedding' studies measure virus shed from infected cells, they do not actually measure virus that is deposited into the touched or respired environment i.e. they do not define environmental contamination and the hazard posed to others. Whilst such data are useful, if they could be linked to near-patient environmental sampling, estimates of the extent to which infectious virus is deposited onto surfaces and into the air in the subject's immediate vicinity could be made.

The occurrence of the 2009/10 influenza pandemic afforded an opportunity to conduct fieldwork and a study was performed to collect data on conventional viral shedding and environmental contamination (surfaces and air) and to investigate the relationships between them.

4.3 Methods

A multi-centre, prospective, observational cohort study recruited subjects over two influenza seasons; September 2009 - January 2010 (Year 1) & December 2010 - January 2011 (Year 2) in accordance with the principles of the Declaration of Helsinki and UK regulatory requirements. It was approved by Leicestershire, Northamptonshire & Rutland Research Ethics Committee 1 (09/H0406/94).

4.3.1 Research Objectives

The primary objectives were to correlate the amount of nasal viral shedding from a subject with; i) the amount of virus recovered from the environment around the subject and ii) symptom duration and severity. Secondary objectives were to describe viral shedding according to patient sub-groups; adults versus children and those with mild illness (community subjects) versus those with more severe disease (hospitalised subjects).

4.3.2 Participants

Subjects who had symptoms suggestive of influenza were recruited from the following groups;

- i) Adults in hospital (AH)
- ii) Children in hospital; age >1 month up to 16 years (CH)
- iii) Adults in the community (AC)
- iv) Children in the community; age >1 month up to 16 years (CC)

The designation AH and CH denote that the subject was enrolled during hospital admission. However, subjects discharged from hospital before the end of follow up were then seen in the community; so whilst initial environmental specimens will have been taken in hospital, later ones will be from the subject's home. No subjects initially enrolled in the community were subsequently admitted to hospital.

4.3.3 Sampling Frames

- Hospital: All cases of suspected influenza identified to researchers by clinical care teams who had agreed to be approached by a researcher. Hospitals involved in recruitment were; Queens Medical Centre and City Hospital, Nottingham; Leicester Royal Infirmary (AH, Year 1 only); Royal Hallamshire Hospital, Sheffield (AH, Year 1 only).

- Community: Individuals living in the Nottingham area who had; i) symptoms of influenza infection, ii) received an invitation to take part in the research and iii) had use of a telephone. Invitations to take part (Appendix 4.1) were given by the following methods;
 - Year 1; adverts in local newspapers, posters sited in community areas, 3000 posted leaflets, 15,000 letters given to parents via schools and 3000 invitations given out at antiviral collection points in areas covered by Nottingham City and Nottinghamshire County PCTs.
 - Year 2; members of staff at two Nottingham NHS walk-in centres, Queens Medical Centre A+E department and a local GP practice.

4.3.4 Eligibility Criteria

Inclusion criteria: A history of influenza-like illness (ILI) defined as;

- Fever (or recent history of fever) + any one of cough, sore throat, runny nose, fatigue or headache OR
- Any two of cough, sore throat, runny nose, fatigue or headache. These symptoms were those most commonly reported by the first cases of A(H1N1)pdm09 in the UK (McLean *et al*, 2010).

Exclusion criteria: Illness present for >48 hours (community cases) or >96 hours (hospital cases); PCR negative for influenza, participation in influenza research involving an investigational medicinal product within the last 3 months (See Appendix 4.2).

Eligible subjects were enrolled after informed consent had been obtained. A subject was defined as a case if; i) they met our criteria for ILI, and ii) tested PCR positive on a nasal swab for influenza. See Appendices 4.3 for participant information sheets and 4.4 for informed consent forms.

4.3.5 Study Procedures

Adult subjects were followed for up to 15 days from the start of symptoms and children <13 years of age were followed for up to 12 days. Where possible, subjects were followed up on a daily basis. Follow up was generally shorter in Year 2 but the surface sampling performed was more intensive, i.e. every day as opposed to alternate days (Table 4.1). This was based on an assumption that environmental contamination is likely to be higher earlier in the course of illness. Data concerning body temperature, medications and room conditions (temperature and humidity) were collected. In addition a symptom diary was completed by each subject; symptoms were given a severity score on a scale of 0-3 (Appendix 4.5).

The following samples were collected;

- Nasal swabs - Whilst a nasopharyngeal aspirate (NPA) is considered the best specimen for detecting influenza viruses (Sung *et al*, 2008; Spyridaki *et al*, 2009; Ngaosuwankul *et al*, 2010), this procedure causes more discomfort and is more difficult to perform, particularly in children. Indeed studies attempting to collect daily NPA samples from subjects have reported problems with subjects' tolerance and compliance with the procedure (To *et al*, 2010). A nasal swab however, has been shown to be an acceptable alternative that is not statistically less sensitive than a NPA (Heikkinen *et al*, 2001; Sung *et al*, 2008; Spyridaki *et al*, 2009).

A rapid antigen test (Quidel Quickvue A+B) using a nasal swab was performed on the day of enrolment to aid the identification of subjects.

Table 4.1: Study procedures conducted in Year1 and Year 2.

Materials and methods	Year 1	Year 2
Timing	Sept 2009 – Jan 2010	Dec 2010 – Jan 2011
Recruitment <ul style="list-style-type: none"> • Community • Hospital 	<p>Antiviral collection points, local media, letters via schools</p> <p>Nottingham, Leicester, Sheffield</p>	<p>NHS walk in centres, A+E department, university campus GP</p> <p>Nottingham</p>
PCR testing to confirm diagnosis performed?	No	Yes
Mean Follow up period	8.7 days	4.8 days
Swabs <ul style="list-style-type: none"> • Hospital • Community 	<p>Cotton tipped</p> <p>Table, window sill, bed button</p> <p>Kettle, tap, door handle, bed table, TV remote, dining table</p>	<p>Dacron tipped</p> <p>Table, cup, bed button, door handle</p> <p>Kettle, tap, door handle, light switch, fridge, TV remote, computer</p>
Frequency	Alternate days	Most days
Air sampling VTM volume	<p>Performed at both 3-7 and >7ft from subject and for 1 and 3 hours</p> <p>750µl VTM was added to samples after collection</p>	<p>Performed at >7 ft from subject for 3 hours</p> <p>1.5mL VTM was added to samples after collection</p>

- Surface swabs - Swabs were taken in a subject's hospital room and/or their own home. Swabs were moistened with VTM and then rubbed across an area of approximately 100cm² in three different directions whilst applying even pressure. In addition to using swabs, sponges were trialled in Year 1 to sample bedside tables. The sponges (TS/15-B:PBS; Technical Service Consultants Ltd) were 50cm² in size, sterile and dosed with 10mL of a neutralising buffer. They were wiped over an approximate 400cm² area and then sealed in a sterile medical grade plastic bag. No specific cleaning instructions were given to households and hospital cleaning continued as normal during follow up. If other household members became ill during the period of follow up, sampling of the original participant continued and the age and symptoms of any potential secondary cases were recorded.

In Year 1 cotton tipped swabs (FB57835; Fisherbrand) were used and in Year 2 Dacron tipped swabs (FB57833; Fisherbrand) were used [this change was made in line with advice to use synthetic fibre swabs for influenza diagnostics (CDC 2009d)]. Swabs and sponges were placed into VTM and kept on 'wet' ice for no longer than 3 hours before being delivered to a laboratory or frozen at -70°C.

- Air particles were collected using a National Institute for Occupational Safety and Health (NIOSH) two-stage cyclone bioaerosol sampler which has been validated for use with influenza (see Chapter 3). The flow rate through each sampler was set at 3.5L/min with a flow calibrator (Model 4143, TSI) before use. Samplers were mounted on tripods at a height of 150cm, were placed at distances of either 3-7 or >7ft from the subject and ran for 1, 2 or 3 hours. Not all subjects were stationary during the sampling period (though they were asked to

remain in the same room if they could), so that the distance from the subject to the sampler may have varied over time. Sampling was usually performed on just one follow up day. After sampling, VTM was added to both stage 1 and 2 tubes and the filter paper was immersed in a 15mL tube also containing VTM. The volumes of VTM were increased in Year 2 to allow sufficient volume for testing. Samples were stored at -70°C .

4.3.6 Laboratory Methods

The following sample processing 'rules' were instituted to limit the analysis of likely negative samples;

- Nasal swabs from Day 4 onwards were not tested if days 1-3 were all PCR negative.
- Culture was only performed on PCR positive samples.
- Environmental swabs were not processed if nasal swabs taken on the three previous days from a case were PCR negative (Year 1) or if nasal swabs were negative on the same day (Year 2).

Laboratory work was carried out at following institutions;

- The HPA and University of Cambridge virology laboratories at Addenbrooke's Hospital, Cambridge. Samples were defrosted and split into six aliquots, three for PCR and three for culture, then refrozen at -70°C until required.
- Queens Medical Centre, Nottingham – Diagnostic PCRs were performed on nasal samples taken on the day of enrolment in Year 2 in order to limit the follow up of influenza negative subjects.

PCR: A novel influenza A H1N1 pentaplex assay was devised to detect virus genome in the samples. The assay was designed to detect A(H1N1)pdm09, seasonal H1 and H3 influenza A, influenza B and the internal control MS2. Viral load data were generated using the PCR assay and plasmids containing the gene target to create a standard curve, such that the concentration of genome present in each sample could be calculated (see Appendix 4.6A for further detail).

Culture: Influenza A(H1N1)pdm09 did not readily form plaques on MDCK cells so an immunofluorescence (IF) assay was used to detect the influenza A/B nucleoprotein in order to demonstrate the presence of live replicating virus in the swab samples. Assays were performed on samples that were PCR positive. Generally, if a swab was IF positive on a given day (e.g. study Day 5) then an assumption was made that previous days (e.g. 1-4) would also have been positive and no testing on these days was done (see Appendix 4.6B for further detail).

4.3.7 Statistical methods

- Sample size

The recruitment target was 100 subjects in total, comprising approximately 25 subjects in each of the four groups. Sub-group sizes of 25 [which allow pooling of data by adults or children (50 per group) or the whole population] gives high statistical power (>80%) to detect correlations of >0.55 in groups of size $n = 25$, 0.4 in groups of size $n = 50$, and 0.3 in groups of size $n = 100$.

- Outcome Measures

1. Viral shedding (nose swab) and environmental deposition (surfaces and air) as measured by PCR and culture techniques.

- A positive nose swab was defined as a sample in which a Ct value of <35 (2342 copies/mL) for ≥ 1 triplicate of a sample is obtained. Implausible results and any single triplicates separated by >48 hours from other positive samples were not counted.
- A positive fomite swab / air sample was defined as a sample in which a Ct value of <35 for ≥ 1 triplicate of a sample is obtained. Post-hoc it was considered that a Ct value of <40 (122 copies/mL) for ≥ 1 triplicate of a sample, if it was obtained from a subject with a positive nose swab taken on the same day, would also be defined as positive. Lowering the threshold of detection to limit falsely negative results was considered reasonable based on a belief that false positive samples were unlikely because; i) 73% (11 out of 15) of subjects had >1 positive sample; and ii) in Year 1 when many more fomite samples from later in the course of a subject's illness were processed, the false positive rate was low (0.6%).
- Viral loads represent the mean value of the triplicate assay. A value of half of the lower limit of detection (i.e. 1171 copies/mL for nose swabs and 61 copies/mL for fomite swabs and air samples) was imputed for undetectable values.
- The duration of viral shedding is defined as the time between symptom onset and the last day that a positive specimen was taken. Because subjects were seldom recruited on the day symptoms began an assumption has been made that they were shedding virus from the first day of symptoms to the last positive specimen.

2. Daily symptom scores categorised into;

- URT score - stuffy nose, runny nose, sneezing, sore throat, sinus tenderness, earache.

- LRT score - cough, shortness of breath.
- Systemic score - fatigue, myalgia, headache.
- Total symptom score is the sum of URT, LRT and systemic symptom scores plus a score for diarrhoea and a score for vomiting.

Scores for each symptom range between 0 and 3. Each symptom score within a category is summed to give an overall category score, for example; cough – 2, shortness of breath – 1 = LRT score of 3. A similar index has previously been used to assess respiratory tract illness of viral aetiology (Jackson *et al*, 1962).

- Analyses

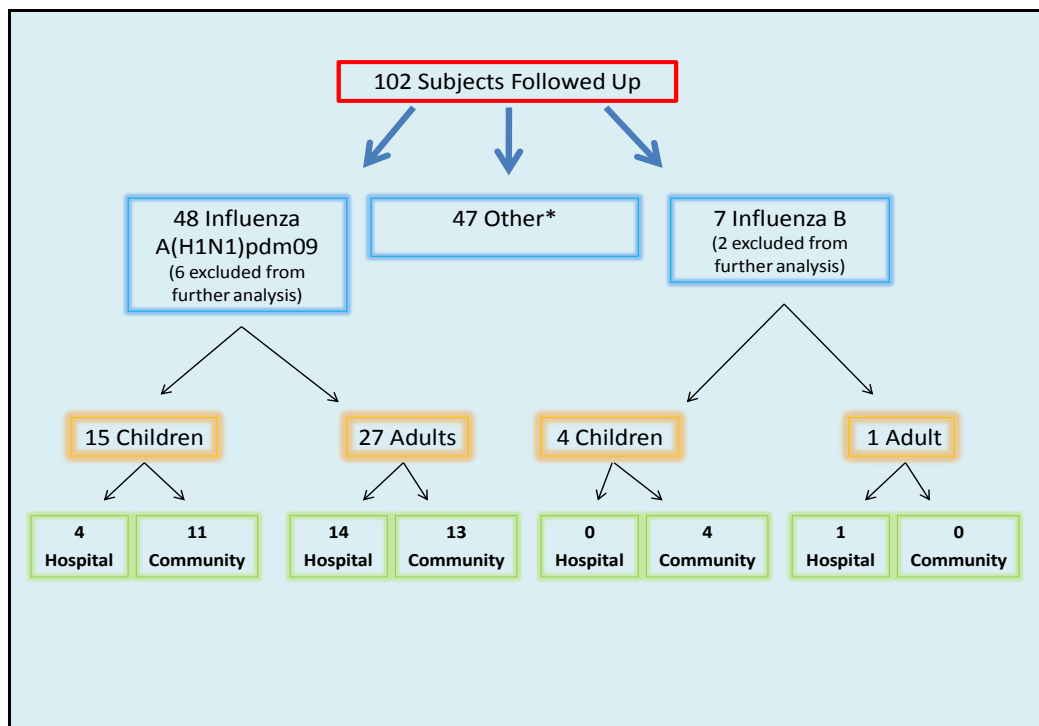
A detailed descriptive analysis of the data is presented. The Student t-test was used to compare mean values. The Pearson's correlation test was used to test correlations between continuous variables and ORs and 95% CI were calculated to represent associations between variables for binary categorical outcome measures. The chi-squared test was used to test the significance of ORs. Differences in viral loads were measured using geometric means (GMs) and compared using geometric mean ratios and the paired t test. P values of ≤ 0.05 were considered significant. All statistical analyses were conducted in Stata version 11.

4.4 Results

4.4.1 Demographics

102 subjects were followed up between September 2009 and January 2011. Influenza A was detected in 48 (47%) subjects; all were A(H1N1)pdm09. Influenza B was detected in seven (7%). Four influenza A cases and two influenza B cases were excluded as study laboratory (as opposed to clinical care) tests were negative. In addition two influenza A cases were excluded on the basis of being recruited >5 days after symptom onset (Figure 4.1).

Figure 1: Participant flow diagram.



*Footnote: *Others consisted of ILI with no confirmed viral aetiology or confirmed infections with rhinovirus and RSV. Four influenza A(H1N1)pdm09 cases and two influenza B cases were excluded as study laboratory (as opposed to clinical care) tests were negative. In addition two influenza A(H1N1)pdm09 cases were excluded on the basis of being recruited >5 days after symptom onset.*

The following results all pertain to the A(H1N1)pdm09 cases unless stated. Of 42 analysed cases, 24 (57%) were female, 27 (64%) were adults and 24 (57%) were community cases. Subjects were recruited after a mean of 2.1 days of illness (Year 1 = 1.8, Year 2 = 2.3) (median = 2, range = 0-4) and were followed for a mean of 6.6 days (Year 1 = 8.7 Year 2 = 4.8) (median = 7, range = 1-12). Seventeen subjects (40%) reported co-morbidities; in ten cases (59%) these included respiratory conditions. The numbers enrolled and a demographic description of A(H1N1)pdm09 cases is shown in Table 4.2.

Table 4.2: Numbers enrolled and overall demographic description of subjects with A(H1N1)pdm09.

	Adult community	Adult hospital	Child community	Child hospital	Total (%)
Enrolled	13	14	11	4	42
Male sex (%)	5	5	8	0	18 (43%)
Median age (yrs)	29	28	4	2.5	22
Range	21-58	19-57	2-12	0-15	0 - 58
Ethnic group					
- White	10	8	8	4	30 (71%)
- Black	1	1	0	0	2 (5%)
- Asian	2	5	1	0	8 (19%)
- Other	0	0	2	0	2 (5%)
Mean time from symptom start to enrolment (days)	2.2	2.4	1.5	2.8	2.1

Tables 4.3A&B list the 42 cases of A(H1N1)pdm09 recruited into the study and shows some of the key outcome measures for each. Two recruited cases needed high dependency care, none died during follow up.

Table 4.3A: A(H1N1)pdm09 positive cases in Year 1.

Subject	Age (years)	Sex (M/F)	Ethnicity	Co-morbidity	Peak total symptom score (DoI / DoFU)	Peak viral load x10 ⁴ copies/mL (DoI / DoFU)	Duration of viral shedding by PCR+ (tested up to day)	Last day culture positive by IF+ (tested up to day)	Day of illness oseltamivir begun#
AC01	21	F	Asian	-	6 (3/1)	32.7 (3/1)	6 (10)	5 (6)	-
AC04	28	F	Black	Asthma	13 (2/1)	8.2 (2/1)	3 (9)	3 (3)	-
AH01	19	F	White	CF	13 (3/1)	5.2 (8/6)	9 (10)	-ve (9)	3
AH03	27	F	Asian	-	28 (3/1)	60.7 (3/1)	9 (12)	4 (9)	2
AH04	30	F	Asian	-	17 (3/1)	1595.9 (8/6)	10 (10)	8 (10)	2
AH05	24	F	Asian	-	12 (4/1)	1.9 (5/2)	5 (7)	-ve (5)	-
AH07	34	M	Asian	-	20 (8/6)	15.4 (4/2)	5 (10)	4 (5)	NA
AH08	33	M	Black	Asthma	25 (3/1)	0.3 (3/1)*	3 (7)	-ve (3)	2
CC01	12	M	Mixed	Asthma	18 (3/1)	3.6 (3/1)	5 (12)	-ve (5)	2
CC02	11	M	Asian	-	18 (3/1)	116.1 (3/1)	8 (11)	-ve (8)	-
CC03	6	M	Asian	-	5 (4/2)	25.1 (3/1)	6 (12)	4 (7)	4
CC04	2	M	White	-	10 (2/1)	1.9 (3/2)	4 (4)	-ve (4)	-
CC05	9	M	White	Asthma	23 (2/1)	1302.5 (2/1)	7 (11)	3 (9)	2
CC06	4	M	White	Eczema	8 (2/2)	15.1 (1/1)	9 (12)	5 (6)	-
CC07	3	F	White	-	8 (2/1)	0.4 (3/2)	3 (10)	3 (3)	2
CC14	6	M	White	-	12 (2/1)	346.6 (2/1)	7 (12)	6 (7)	-
CC15	2	F	White	-	10 (6/4)	2452.1 (3/1)	8 (13)	-ve (8)	-
CH01	15	F	White	-	10 (3/1)	286.5 (3/1)	8 (10)	6 (8)	3
CH03	0	F	White	CF	4 (4/1)	1847.5 (5/2)	7 (10)	5 (7)	4

Table 4.3B: A(H1N1)pdm09 positive cases in Year 2.

Subject	Age (years)	Sex (M/F)	Ethnicity	Co-morbidity	Peak Total Symptom Score (DoI / DoFU)	Peak Viral Load x10 ⁴ copies/mL (DoI / DoFU)	Duration of viral Shedding by PCR [†] (tested up to day)	Last Day Culture Positive by IF [†] (tested up to day)	Day of illness oseltamivir begun [‡]
AC20	43	M	White	-	10 (3/2)	340.2 (4/3)	6 (6)	4 (6)	-
AC21	58	F	White	DM	21 (3/2)	27.2 (3/2)	5 (7)	5 (5)	-
AC22	23	M	Asian	-	18 (3/1)	70.2 (3/1)	6 (8)	3 (3)	-
AC26	45	F	White	-	32 (5/3)	95.1 (3/1)	5 (8)	3 (5)	-
AC28	22	F	White	-	26 (4/2)	773.5 (4/2)	4 (7)	4 (4)	-
AC29	21	F	Black	Asthma	18 (5/1)	1.8 (6/2)	10 (10)	10 (10)	-
AC30	25	M	White	-	11 (3/1)	316.6 (4/2)	7 (7)	4 (7)	-
AC32	57	M	White	-	18 (3/1)	8.0 (3/1)	4 (4)	-ve (4)	-
AC33	33	F	White	-	15 (5/1)	1.9 (5/1)	6 (6)	-ve (6)	-
AC36	30	M	White	-	15 (3/1)	6.8 (3/1)	7 (9)	ND	-
AC37	22	F	White	Asthma	22 (6/3)	55.4 (6/3)	6 (10)	6 (6)	-
AH20	52	M	White	-	18 (4/1)	258.4 (4/1)	15 (15)	5 (13)	4
AH24	31	F	White	Asthma	18 (3/1)	173.5 (3/1)	4 (8)	4 (4)	3
AH26	57	F	White	CCF, DM	4 (4/1)	2381.8 (4/1)	6 (7)	4 (7)	4
AH27	42	M	White	IHD	20 (4/1)	417.1 (4/1)	6 (6)	-ve (6)	3
AH28	22	M	White	-	10 (4/1)	33.4 (4/1)	5 (5)	-ve (5)	4
AH29	19	F	White	Asthma	28 (3/1)	1120.5 (3/1)	4 (4)	3 (4)	1
AH32	31	F	White	-	22 (5/3)	5.5 (4/2)	5 (10)	-ve (5)	4
AH42	37	F	Asian	-	14 (4/1)	60.5 (4/1)*	4 (5)	-ve (4)	3
CC25	3	M	White	-	16 (4/1)	1.2 (4/1)	5 (7)	-ve (5)	-
CC26	3	F	White	-	6 (2/1)	15.0 (2/1)*	2 (2)	-ve (2)	-
CH21	5	F	White	Eczema	15 (5/1)	0.3 (5/1)	10 (10)	ND	-
CH30	0	F	White		12 (6/4)	1.2 (8/6)	7 (9)	ND	4

Footnote: AC = Adult Community, AH = Adult Hospital, CC = Child Community, CH = Child Hospital, DoI = Day of Illness (= day of follow up + time to enrolment; Day 1 is the day symptoms were first noticed), DoFU = Day of Follow Up, CF = Cystic Fibrosis, DM = Diabetes Mellitus, CCF = Congestive Cardiac Failure, ND = Not Done, NA = Not Available, * Only data on one viral load available, † Time from symptom onset to last day swab positive. ‡ First day of symptoms = Day 1, therefore treatment at Day 3 is within 48 hours

4.4.2 Rapid antigen tests

Sixteen out of 40 (40%) subjects were rapid antigen test positive; 8 out of 27 (30%) adults and 8 out of 15 (53%) children. There were no false positives tests.

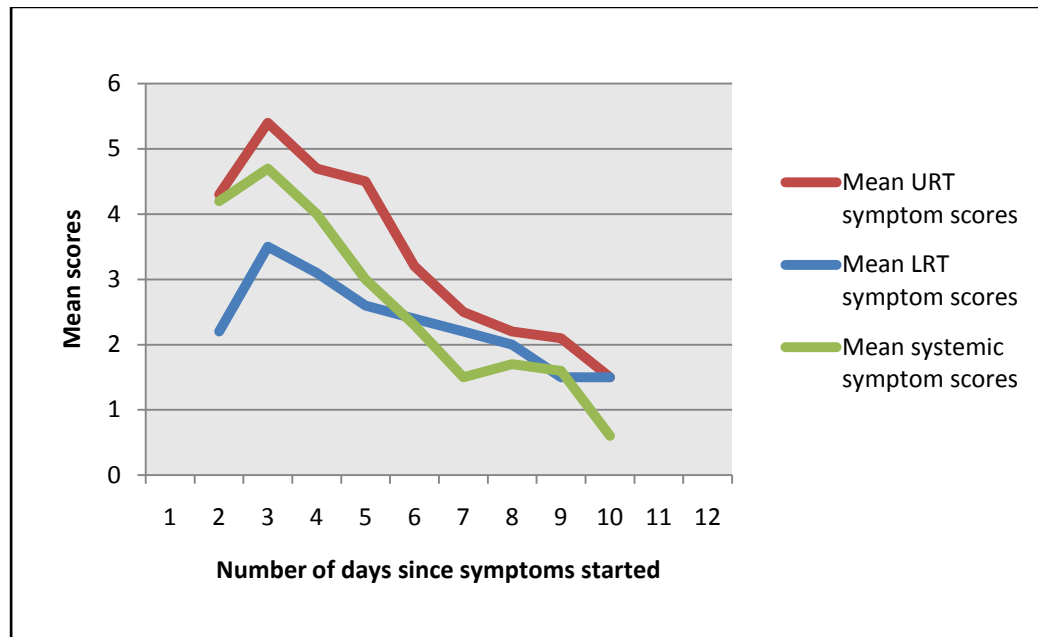
4.4.3 Symptoms

The most frequently reported symptoms were cough, sore throat and runny nose (Table 4.4). Fever was reported on the day illness began in 21 out of 42 (50%) cases and was measured as high ($\geq 38^{\circ}\text{C}$) during follow up in 14 out of 42 (33%) of cases. During follow up symptom scores were highest on Day 3 of illness and declined thereafter (Figure 4.2).

Table 4.4: Symptoms reported over the course of study follow up.

Symptom	A(H1N1)pdm09 Subjects (n=42)
Fever (on day of onset)	50%
Cough	93%
Sore throat	88%
Runny nose	86%
Stuffy nose	81%
Fatigue	76%
Sneezing	74%
Headache	71%
Myalgia	55%
Shortness of breath	52%
Sinus Tenderness	52%
Vomiting	31%
Earache	26%
Diarrhoea	14%

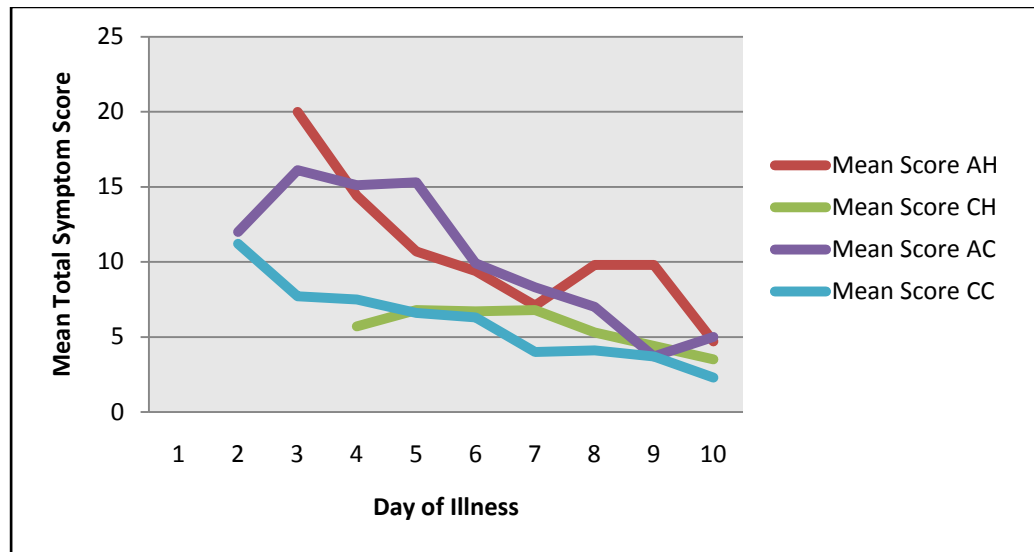
Figure 4.2: Mean symptom scores of A(H1N1)pdm09 cases over time.



Footnote: Data only shown where ≥ 3 observations are available.

Children had lower symptom scores than adults (Figure 4.3). When matched for study setting, total symptom and systemic scores on Days 3 and 4 were significantly lower in children compared to adults (except for total symptom scores in hospital cases on Day 3). There were no consistent differences in relation to symptom scores between community and hospital subjects on illness Days 3 or 4 (Tables 4.5 & 4.6).

Figure 4.3: Comparison of mean total symptom scores over time.



Footnote: Data only shown where ≥ 3 observations are available.

Table 4.5: Symptom score data.

	All Adults	All Children	All Community	All Hospital	AH	CH	AC	CC
n	27	15	18	24	14	4	13	11
Mean TS Day 3	17.8	8.1	12.1	18.0	20	10	16.1	7.7
Mean TS Day 4	14.7	7.1	11.3	12.9	14.4	5.7	15.1	7.5
Mean LRT Day 3	4.1	2.6	2.79	4.8	5.0	4.0	3.3	2.2
Mean LRT Day 4	3.6	2.1	2.8	3.4	3.7	1.7	3.4	2.2
Mean URT Day 3	6.8	3.2	4.9	6.5	7.4	3.0	6.4	3.2
Mean URT Day 4	5.4	3.3	4.8	4.5	5.1	2.0	5.8	3.8
Mean SS Day 3	6.6	1.7	4.2	5.8	6.8	2.0	6.4	1.7
Mean SS Day 4	5.3	1.4	3.7	4.2	4.9	1.0	5.7	1.6

Footnote: TS = Total Symptoms, URT = Upper Respiratory Tract, LRT = Lower Respiratory Tract, SS = Systemic Symptoms, AC = Adult Community, AH = Adult Hospital, CC = Child Community, CH = Child Hospital

Table 4.6: Symptom score analyses.

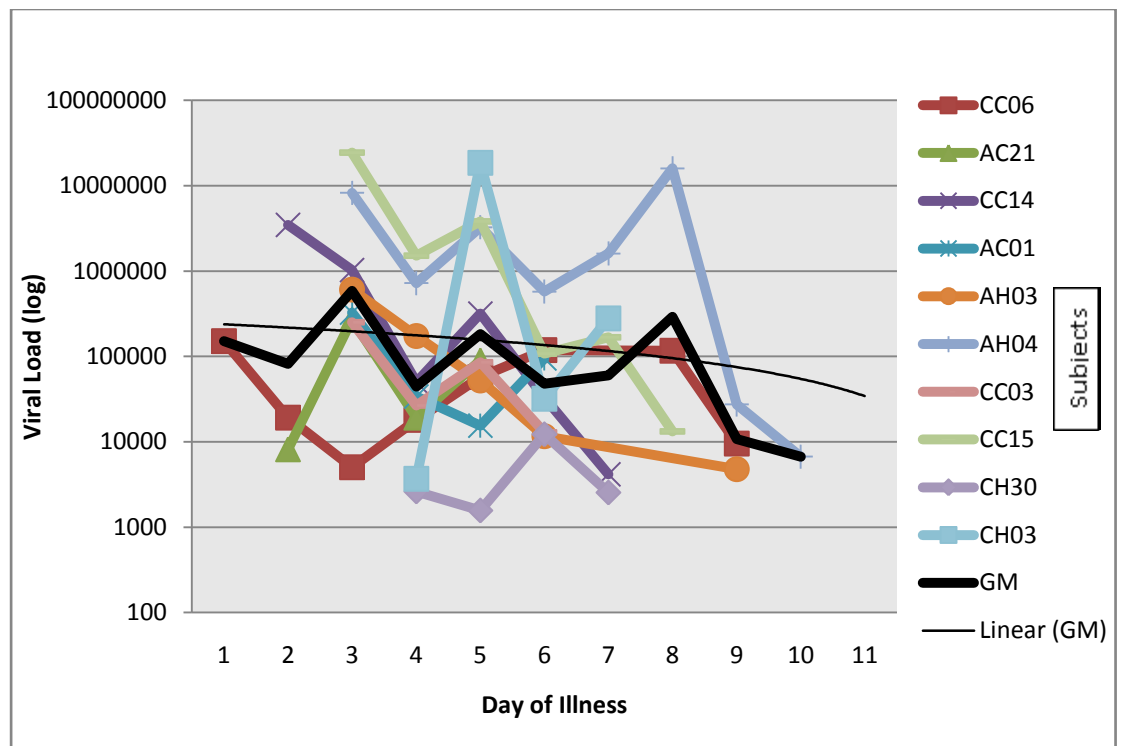
	Adults vs. Children	Community vs. Hospital	CH vs. AH	CC vs. AC	CC vs. CH	AC vs. AH
TS Day 3 MD (95% CI) P value	-9.74 (-14.8, -4.7) 0.0005	-5.89 (-11.9, 0.1) 0.055	-10 (-20.6, 0.6) 0.06	-8.4 (-14.9, -2.0) 0.01	-2.33 (-12.4, 7.8) 0.61	-3.9 (-10.7, 2.9) 0.24
TS Day 4 MD (95% CI) P value	-7.63 (-12, -3.4) 0.0008	-1.58 (-6.3, 3.2) 0.50	-8.7 (-16.4, -1.1) 0.03	-7.6 (-13.8, -1.4) 0.01	1.83 (-3.6, 7.3) 0.47	0.67 (-5.4, 6.8) 0.82
LRT Day 3 MD (95% CI) P value	-1.51 (-2.7, -0.3) 0.018	-2.01 (3.2, -0.9) 0.0012	-1.0 (-3.4, 1.4) 0.37	-1.08 (-2.4, 0.3) 0.11	-1.78 (-3.6, 0.4) 0.055	-1.7 (-3.3, -0.2) 0.03
LRT Day 4 MD (95% CI) P value	-1.5 (-2.7, -0.4) 0.0072	-0.5 (-1.6, 0.6) 0.35	-2.05 (-4.3, 0.2) 0.07	-1.18 (-2.6, 0.2) 0.08	0.56 (-0.6, 1.7) 0.32	-0.31 (-1.8, 1.2) 0.67
URT Day 3 MD (95% CI) P value	-3.65 (-6.5, -0.8) 0.0143	-1.61 (-4.8, 1.6) 0.3136	-4.38 (-11.9, 3.1) 0.22	-3.18 (-6.7, 0.3) 0.07	0.22 (-3.7, 4.1) 0.90	-0.98 (-5.4, 3.5) 0.65
URT Day 4 MD (95% CI) P value	-2.04 (-4.3, 0.2) 0.0751	0.31 (-1.9, 2.6) 0.78	-3.07 (-6.6, 0.5) 0.09	-2.02 (-5.5, 1.5) 0.24	1.78 (-1.2, 4.7) 0.21	0.73 (-2.4, 3.8) 0.63
SS Day 3 MD (95% CI) P value	-4.83 (-6.3, -3.4) <0.0001	-1.64 (-4., 0.7) 0.1667	-4.75 (-7.5, -2.1) 0.004	-4.73 (-6.8, -2.7) 0.0001	-0.33 (-4.4, 3.6) 0.86	-0.35 (-2.1, 1.4) 0.67
SS Day 4 MD (95% CI) P value	-3.83 (-5.6, -2.1) 0.0001	-0.5 (-2.6, 1.6) 0.62	-3.92 (-7.2, -0.7) 0.02	-4.14 (-6.6, -1.7) 0.002	0.56 (-2.4, 3.5) 0.69	0.77 (-1.5, 3.0) 0.48

Footnote: TS = Total Symptoms, SS = Systemic Symptoms, URT = Upper Respiratory Tract, LRT = Lower Respiratory Tract, MD = Mean Difference, AC = Adult Community, AH = Adult Hospital, CC = Child Community, CH = Child Hospital

4.4.4 Viral load

Nasal swab viral loads were examined over time and in relation to symptom scores. Viral loads, measured by PCR, varied widely across our A(H1N1)pdm09 positive subjects, ranging from 2033 – 24,521,397 copies/mL. As shown in Figure 4.4, viral loads declined over time.

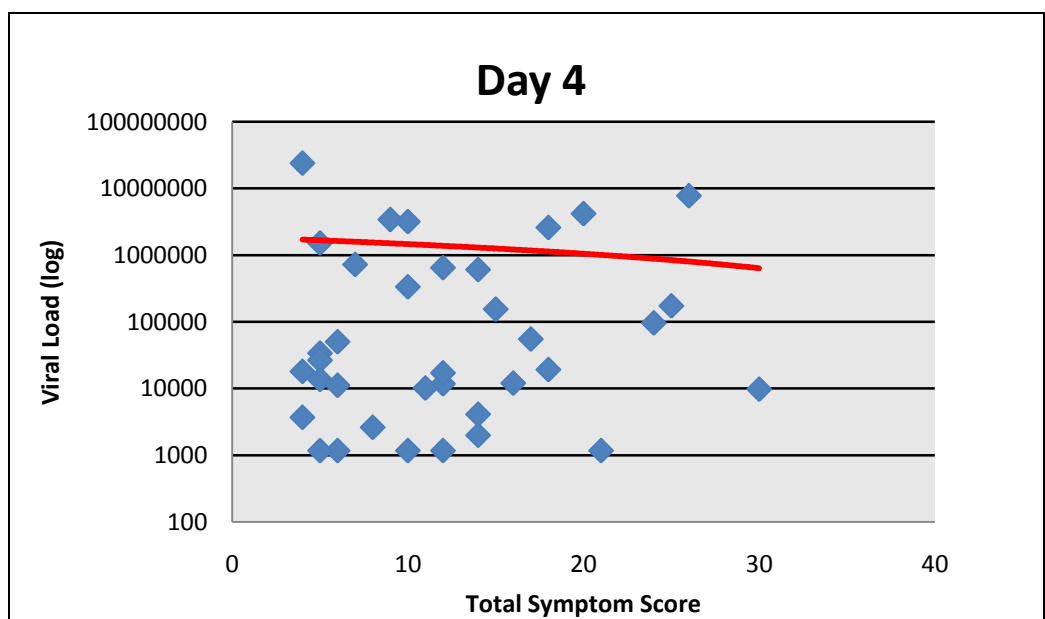
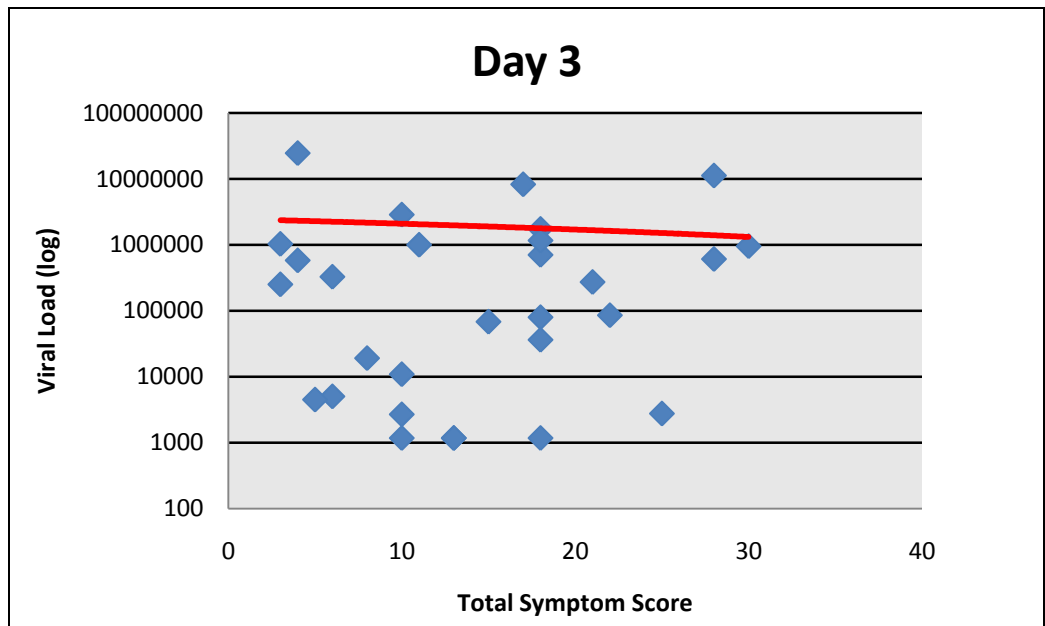
Figure 4.4: Viral Loads over time.



Footnote: Viral loads plotted over time are shown for ten subjects from whom the most complete data were obtained (≥ 4 consecutive values). The geometric mean viral load for each day is represented by GM and its linear trend line is also shown., AC = Adult Community, AH = Adult Hospital, CC = Child Community, CH = Child Hospital

Poor correlations were observed between total symptom scores and viral loads on illness Days 3 ($r = -0.0628$; $p > 0.05$) and 4 ($r = -0.0700$; $p > 0.05$) (Figure 4.5).

Figures 4.5A (top) + 4.5B (bottom): Scatter plots showing the relationship between viral load and total symptom score on illness Day 3 and 4.



Footnote: Linear trend lines are shown in red.

No significant differences were seen in the geometric mean viral loads between adults vs. children and community vs. hospital cases on illness Days 3, 4 and 5 (Table 4.7).

Table 4.7: Geometric mean viral loads compared between groups.

Illness day	GM VL adults (95% CI)	GM VL children (95% CI)	Adult/Children GM ratio (95% CI)	P value
Day 3	121972 (26689, 557430)	132520 (16143,1087878)	0.92 (0.1, 10.4)	0.945
Day 4	98666 (26015, 374210)	20303 (5386, 76532)	4.86 (0.8, 29.1)	0.081
Day 5	31311 (12005, 81663)	26187 (4417, 155248)	1.20 (0.2, 8.1)	0.850
Illness day	GM VL community (95% CI)	GM VL hospital (95% CI)	Hospital/Community GM ratio (95% CI)	P value
Day 3	139051 (44444, 435047)	102360 (4271, 2452955)	0.74 (0.0, 14.7)	0.835
Day 4	36510 (10204, 130630)	104849 (19876, 553104)	2.87 (0.4, 21.3)	0.292
Day 5	16669 (5591, 49698)	56467 (12781, 249478)	3.39 (0.6, 19.7)	0.168

Footnote: GM = Geometric Mean, VL = Viral Load

Amongst sub-groups there was a trend and one significant observation suggesting AH have higher GM viral loads than CH. There was also a significant difference in GMs on illness Day 4 suggesting that CC have higher viral loads than CH (Table 4.8).

Table 4.8: Geometric mean viral loads compared between groups.

	AH vs. CH	AC vs. CC	CH vs. CC	AH vs. AC
Reference category	CH	CC	CC	AC
Day 3 GMs GM ratio (95% CI) P value	120446/57917 2.1 (0.0, 9031.1) 0.842	123051/159280 0.8 (0.1, 8.5) 0.823	57917/159280 0.4 (0.0, 516.8) 0.760	120446/123051 1.0 (0.0, 35.2) 0.990
Day 4 GMs GM ratio (95% CI) P value	180149/3110 57.9 (9.7, 345.8) <0.001	45110/29549 1.5 (0.1, 21.0) 0.738	3110/29549 0.1 (0.0, 0.5) 0.009	180149/45110 4.0 (0.3, 61.8) 0.305
Day 5 GMs GM ratio (95% CI) P value	60106/47556 1.26 (0.0, 116.6) 0.913	12772/20628 0.6 (0.1, 5.2) 0.638	47556/20628 2.3 (0.0, 269.1) 0.709	60106/12772 4.7 (0.8, 26.6) 0.076

Footnote: AC = Adult Community, AH = Adult Hospital, CC = Child Community, CH = Child Hospital, GM = Geometric Mean

4.4.5 Viral shedding

The data on shedding presents a number of difficulties for interpretation.

As a result a number of estimates for shedding have been derived; they are referred to as A, B and C;

- A. Estimate based only on positive results.
- B. Estimate that includes only samples that have a positive followed by a negative result or that were positive for at least 5 days from illness onset. Some positive subjects were only followed for a short period of time with the result that 'A' may underestimate the true duration of shedding. This estimate attempts to describe an upper bound for shedding.
- C. Estimates based on all results - Negative results may arise through false negatives or because the duration of viral shedding was too short

to be observed. Therefore 'A' may overestimate the duration of shedding. To obtain a lower bound for the duration, the calculation is repeated with the assumption that "negative" subjects did not shed live virus (duration of shedding = 0).

The duration of viral shedding is defined as the time between symptom onset and the last day that a positive specimen was taken. Because subjects were seldom recruited on the day symptoms began an assumption has been made that they were shedding virus from the first day of symptoms to the last positive specimen.

- Shedding by PCR

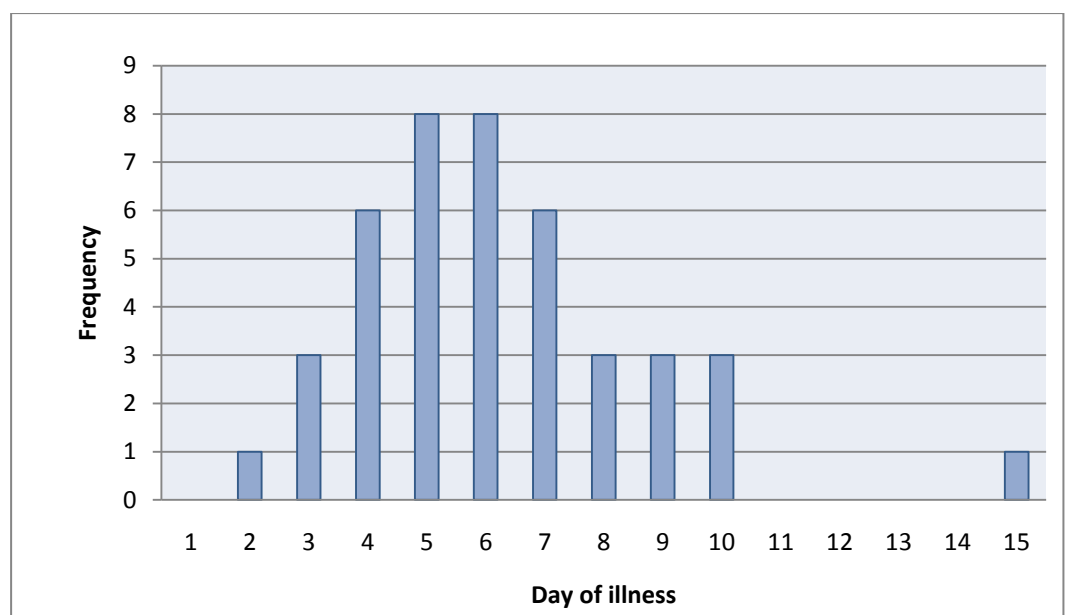
Duration;

A. 6.2 days, range 2-15 days (n = 42) (Figure 4.6)

B. 6.5 days, range 3-15 days (n = 38)

C. N/A (by definition all cases were PCR positive on at least one day)

Figure 4.6: Distribution of the duration of viral shedding by PCR positivity (using estimate A).



Based on estimate A, adults shed virus for 6.11 days (95%CI: 5.08, 7.14) compared to children who shed for 6.40 days (95%CI: 5.17, 7.63); mean difference = 0.29 (95% CI: -1.33, 1.90), $p = 0.72$. There were no differences in the mean duration of shedding between hospitalised adults and children, community adults and children, hospitalised adults and children and community adults and children (Table 4.9).

Table 4.9: Duration of PCR shedding (estimate A) compared between groups.

	All adults	All children	AH	CH	AC	CC
n	27	15	14	4	13	11
Mean duration of shedding	6.1	6.4	6.4	8.0	5.8	5.8
	Adults vs. Children	CH vs. AH	AC vs. CC	CC vs. CH	AC vs. AH	
Mean difference (95% CI)	0.29 (1.3, 1.9)	1.6 (2.0, 5.2)	0.05 (-1.6, 1.7)	-2.2 (-4.8, 0.4)	-0.6 (-2.7, 1.4)	
P value	0.72	0.37	0.95	0.094	0.52	

Footnote: AC = Adult Community, AH = Adult Hospital, CC = Child Community, CH = Child Hospital

Symptom score and duration of shedding;

Mean total symptom scores on Days 3 and 4 of illness were significantly higher in those who shed for <6 days compared to those who shed for ≥ 6 days; no difference was observed on Day 5. Although not significant these

trends largely held when adults and children were analysed separately (Table 4.10).

Table 4.10: Data showing associations between symptom scores and duration of shedding (by PCR).

Illness day	Mean Total Symptom Score (All Subjects)		Mean Difference (95% CI)	P value
	Shedding <6 days (95% CI)	Shedding ≥6 days (95% CI)		
Day 3	18.00 (13.46, 22.54)	11.00 (7.30, 14.70)	7.00 (1.49, 12.51)	0.0148
Day 4	14.52 (10.92, 18.14)	9.90 (6.89, 12.91)	4.63 (0.14, 9.12)	0.0436
Day 5	10.92 (5.65, 16.20)	9.86 (7.01, 12.70)	1.07 (-4.17, 6.30)	0.6811
Illness day	Mean Total Symptom Score (Adults)		Mean Difference (95% CI)	P value
	Shedding <6 days (95% CI)	Shedding ≥6 days (95% CI)		
Day 3	20.30 (15.81, 24.79)	14.75 (9.21, 20.29)	5.55 (0.92, 12.02)	0.0876
Day 4	16.15 (12.03, 20.27)	13.00 (8.26, 17.74)	3.15 (2.73, 9.04)	0.2784
Day 5	12.89 (5.69, 20.08)	12.73 (8.21, 17.24)	0.16 (7.40, 7.71)	0.9647
Illness day	Mean Total Symptom Score (Children)		Mean Difference (95% CI)	P value
	Shedding <6 days (95% CI)	Shedding ≥6 days (95% CI)		
Day 3	10.34 (6.58, 27.24)	7.25 (2.90, 11.60)	3.08 (5.49, 11.66)	0.4370
Day 4	9.25 (0.79, 17.71)	6.11 (4.17, 8.05)	3.13 (1.51, 7.78)	0.1650
Day 5	6.50 (2.73, 15.73)	6.7 (3.88, 9.52)	-0.2 (5.98, 5.58)	0.9411

- Shedding by culture

The duration of viable viral shedding is defined as the time between symptom onset and the last day that a positive culture was obtained.

Cultures were performed in reverse order from the last day of nasal swab PCR positivity; if PCR was positive on study days 3-5, culture would be done first on the sample from Day 5, then Day 4 and then Day 3. If a culture was positive on any given day, then we assumed that earlier days would also have been positive.

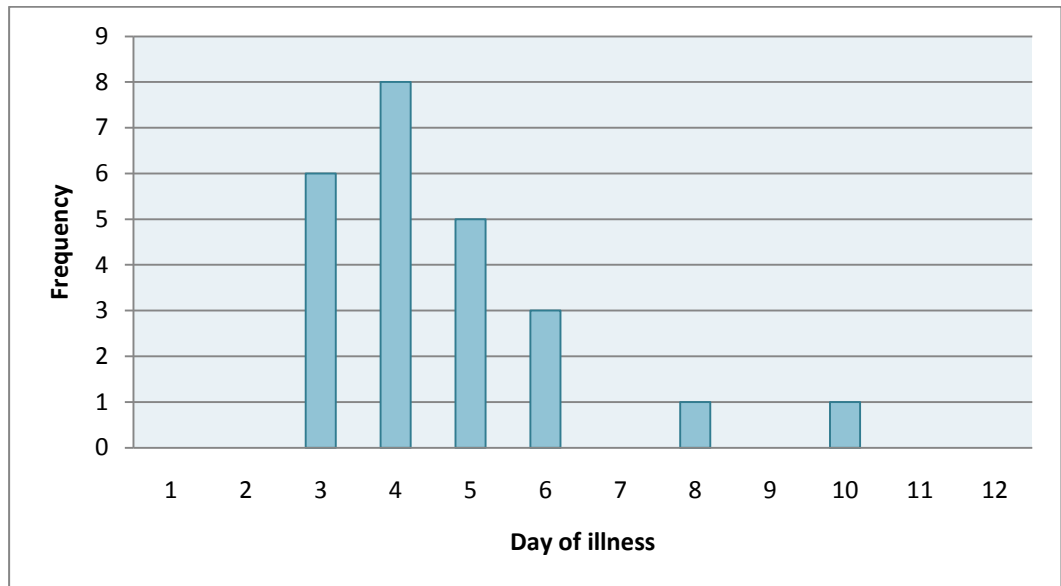
Twenty four out of 39 cases (62%) were culture positive (culture was not performed in three cases due to insufficient sample).

Duration;

- A. Positive results only; mean 4.6 days, range 3-10 days (n = 24) (Figure 4.7)
- B. Samples that have a positive followed by a negative result or that were positive for at least 5 days from illness onset; mean 4.9 days, range 3-10 days (n = 19)
- C. All results; mean 2.8 days, range 0-10 days (n = 39)

Ten out of 39 (26%) subjects shed live virus for at least 5 days from the onset of illness. Based on definition B, the mean duration of shedding was 4.8 and 5.0 days in children and adults respectively; mean difference: -0.2 (95% CI: -2.04, 1.70); p = 0.85.

Figure 4.7: Distribution of the duration of viral shedding by culture positivity (using estimate A).



Symptom score and duration of shedding;

No statistically significant associations were observed between the mean total symptom scores on Days 3, 4 and 5 of illness in those who shed for ≥ 5 days compared to those who shed for < 5 days (Table 4.11).

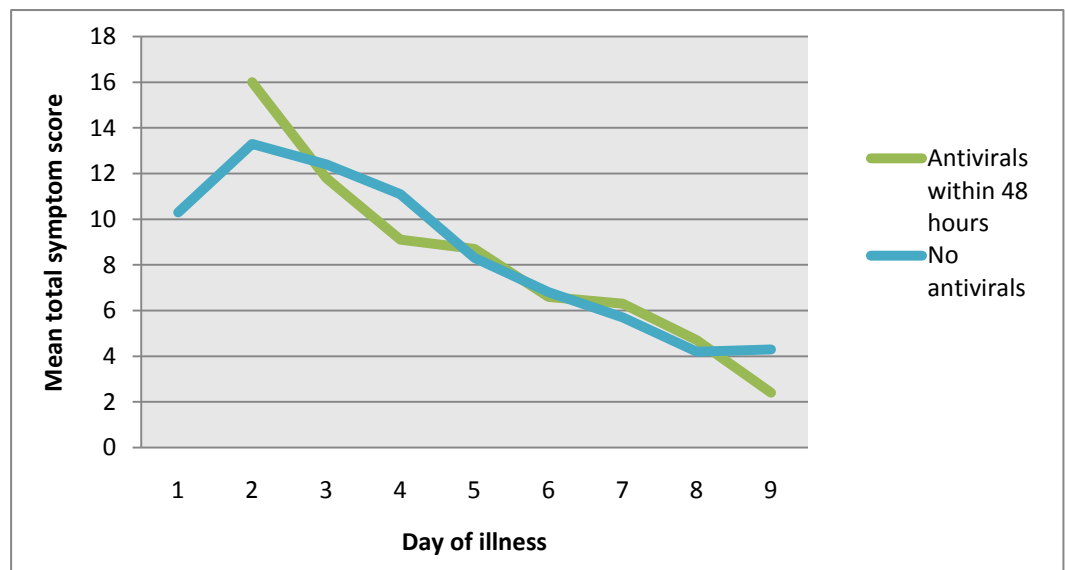
Table 4.11: Data showing associations between symptom scores and duration of shedding (by culture).

Illness day	Mean Total Symptom Score (All Subjects)		Mean Difference (95% CI)	P value
	Shedding < 5 days (95% CI)	Shedding ≥ 5 days (95% CI)		
Day 3	15.3 (11.73, 18.9)	10.5 (3.09, 17.91)	4.8 (-2.7, 12.3)	0.20
Day 4	12.9 (10.14-15.71)	9.8 (4.37-15.18)	3.1 (-2.4, 8.7)	0.25
Day 5	10.5 (7.22-13.83)	9.8 (4.64-14.92)	0.7 (-5.2, 6.7)	0.74

4.4.6 Antivirals

Twenty out of 42 (48%) received an antiviral (all oseltamivir); hospital cases 16 out of 18 (89%), community cases 4 out of 24 (17%), adults 13 out of 27 (48%), children 7 out of 15 (46%). Twelve out of 41 (29%) took oseltamivir within 48 hours (data on when treatment was begun for one subject is not available). In Year 1, 4 out of 11 (36%) community cases received oseltamivir compared to 0 out of 13 (0%) in Year 2. There appeared to be no difference in the rate of symptom decline between those who took antivirals within 48 hours and those who did not take antivirals at all (Figure 4.8).

Figure 4.8: Symptoms scores over time for those who took antivirals within 48 hours and those who did not take antivirals.



Footnote: Only points shown where ≥ 3 data points available

Durations of viral shedding by group of antiviral exposure is shown in Table 4.12. No significant differences were observed for the mean differences between the groups who took antivirals and the group who did not (Table 4.13).

Table 4.12: Duration of shedding by antiviral exposure (using estimate B).

	Took antivirals	Took antivirals within 48 hours	Did not take antivirals
Shedding by PCR	6.53 (n=18)	6.18 (n=11)	6.47 (n=19)
Shedding by culture	4.6 (n=10)	4.8 (n=5)	5.33 (n=9)

Table 4.13: Mean Differences in shedding between antiviral exposure groups.

	AV vs. NoAV	AV48 vs. NoAV
Mean Difference in shedding by PCR (95% CI)	-0.05 (-1.65, 1.54)	0.29 (-1.38, 1.97)
P value	0.95	0.72
Mean Difference in shedding by culture (95% CI)	0.73 (-0.97, 2.44)	0.53 (-1.97, 3.03)
P value	0.38	0.65

4.4.7 Symptom and viral shedding data summary

- During follow up the highest symptom scores were recorded on illness Day 3.
- Adults reported more symptoms than children/parents of children.
- There was a trend to higher viral loads in hospitalised compared to community cases.
- No clear relationship between symptom scores and viral load was evident.
- The typical duration of PCR detectable viral shedding was 6-7 days.
- In those who were culture positive the mean duration of viable viral shedding was 4-5 days.
- No clear distinction in the duration of viral shedding between adults and children or community and hospital cases was shown.

4.4.8 Environmental Deposition

➤ Surfaces

662 samples were collected and tested; 651 swabs and 11 sponges.

A(H1N1)pdm09 was detected by PCR from 36 samples (5.4%). Positive

samples were obtained from 15 out of 40 subject locations (38%); >1

positive sample was obtained from 11 subjects. Seventeen samples

(selected on the basis of strongly positive PCR results) were examined for

viable virus and two surfaces from different subjects were positive (Table

4.14).

- Year 1: 413 swabs collected, 356 samples processed (86%), five positive (1.4%). 18 surface samples were collected with sponges, 11 were processed, one was positive (9.1%).
- Year 2: 485 swabs collected, 306 samples processed (63%), 30 positive (10.7%).

Differences between Year 1 and 2;

- Cotton tipped swabs were used in Year 1 and Dacron tipped swabs were used in Year 2.
- The surfaces swabbed were slightly different – more commonly touched and more non-porous surfaces were selected in Year 2 (see Table 1).
- Sample processing - In Year 1 most collected samples were analysed whereas in Year 2 samples were usually only tested when nose swabs were triplicate PCR positive on the same day. If Year 1 samples had been subject to the same rules, then 201 (49%) would have been processed and the positivity rate would have been 4 out of 201 (2.0%).
- Swabbing in Year 2 tended to be performed early in the course of illness. In Year 1 swabbing was evenly spaced over the duration of follow up (which averaged 8.7 days) compared to Year 2 where swabbing tended to be performed on most days of follow up (average 4.8 days).

Table 4.14: Details of surfaces swabs that were positive for A(H1N1)pdm09.

Subject ID	Surface	Material	Setting	Day of illness	VL surface (copies/mL x 10 ⁴)	VL nose (copies/mL x 10 ⁴)
AC21	Remote	Plastic	Home	3	0.07	27.2
	Tap	Metal		4	0.03	1.9
AC22	Tap	Metal	Home	3	1.2	70.2
	Games Console	Plastic			1.2	
AC26	Tap	Metal	Home	3	0.02	95.1
AC28	Fridge	Plastic	Home	4	1.0	773.5
	Tap	Metal			0.04	
AC30	Remote	Plastic	Home	3	0.3	99.9
	Door handle	Metal			0.1	
	Laptop	Plastic		4	1.0	316.6
	Fridge	Plastic			0.4	
	Remote	Plastic			1.1	
	Light switch	Plastic			0.14	
	Light switch	Plastic		7	0.02	2.1
Tap	Metal	0.1				
AC36	Laptop	Plastic	Home	3	0.02	6.8
AC37	Laptop	Plastic	Home	6	0.07	55.4
AH04*	Kettle	Plastic	Home	4	0.5	72.2
	Tap	Metal		10	0.94	0.7
AH08	Table	Veneer	Hospital	3	0.14	0.3
	Table#	Veneer			0.02	
AH20	Table	Veneer	Hospital	4	0.07	258.4
	Cup	Plastic			0.1	
	Cup	Plastic		5	0.2	39.8
	Bed Rail	Metal		9	0.02	2.6
AH24	Table	Veneer	Hospital	3	0.006	173.5
	Remote	Plastic	Home	4	0.01	0.2
	Fridge	Plastic			0.01	
AH29	Bed control	Plastic	Hospital	3	0.42	1120.5
	Table	Veneer			0.27	
CC25*	Chair	Plastic	Home	5	0.03	0.6
	Remote	Plastic			0.01	
CC26*	Light switch	Plastic	Home	2	0.6	15.0
	Tap	Metal			0.5	
	Cup	Ceramic			0.7	
CH01	Bed control	Plastic	Hospital	3	0.17	286.5

Footnote: * Other household members present who were exhibiting respiratory symptoms, # Sponge swab, Culture positive

On illness Day 3 a significantly higher GM viral load was seen in those who had surface positive swabs compared to those who did not. There were also some statistically significant findings and trends to suggest that those who had positive surface swabs taken from their environment were more likely to have higher URT and LRT symptom scores respectively (Table 4.15).

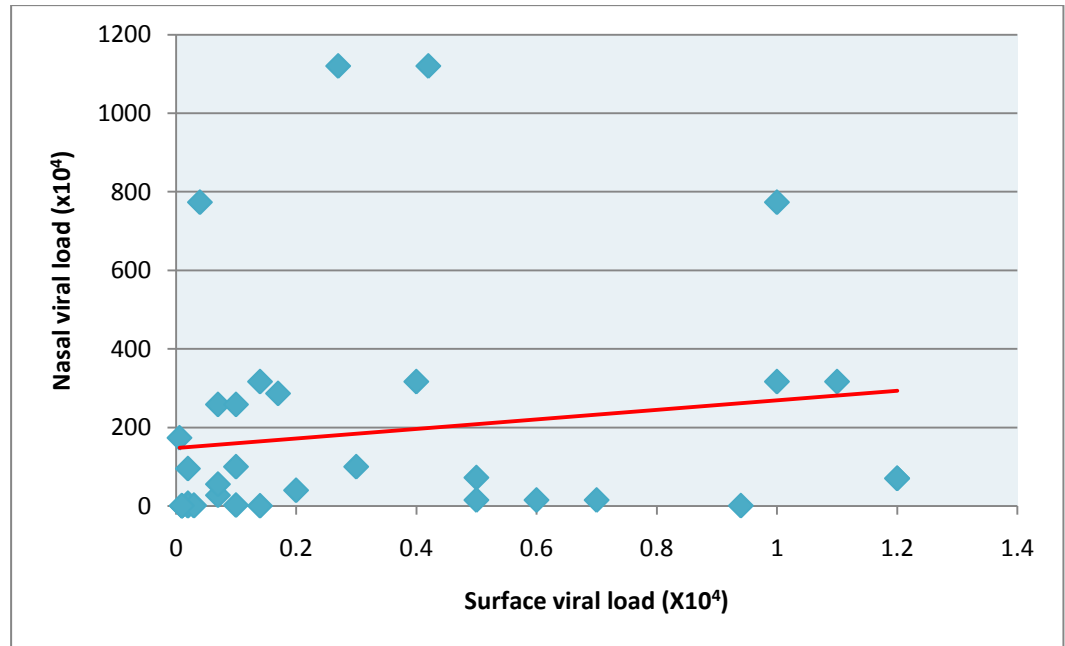
Table 4.15: Viral loads and symptom scores compared between those with positive and those with negative surface swabs.

Illness day	GM nasal VL surface positive (95% CI)	GM nasal VL surface negative (95% CI)	GM ratio	P value
Day 3	464225.5 (79759.9, 2701927.0)	18072.4 (1573.2, 207613.6)	25.7 (1.75, 376.0)	0.021
Day 4	77514.8 (7301.8, 822885.5)	118788.1 (19080.1, 739547.4)	0.7 (0.0, 10.5)	0.753
Illness day	Mean URT score surface positive	Mean URT score surface negative	Mean difference	P value
Day 3	8.5	3.9	-4.6	0.002
Day 4	6.6	3.6	-3.0	0.009
Illness day	Mean LRT score surface positive	Mean LRT score surface negative	Mean difference	P value
Day 3	4.2	3.2	-1.0	0.14
Day 4	3.8	2.7	-1.1	0.051

Footnote: GM = Geometric Mean, VL = Viral Load, URT = Upper Respiratory Tract, LRT = Lower Respiratory Tract

A positive, but non-significant, correlation ($r = 0.1574$, $p > 0.05$) was observed between viral loads recovered from surfaces and nasal viral loads (Figure 4.9).

Figure 4.9: Scatter plot showing relationship between nasal and surface viral loads.



Footnote: Linear trend line shown in red.

➤ Air

Air samples were collected from the immediate environment of 12 subjects (Year 1 = 5, Year 2 = 7); six were in hospital, nine were adults and eight were rapid test positive (Table 4.16). Twenty seven air collections were undertaken generating 81 size fractionated samples (one sample could not be processed because of insufficient sample volume). Positive samples were obtained from five subjects (42%) (Table 4.17); 9 out of 27 (33%) collections and 23 out of 80 (29%) samples were positive for PCR. Viral loads ranged between 238 and 24,231 copies/mL. No samples were culture positive.

Table 4.16: Description of air samples collected.

Subject ID	Any air sample PCR +ve	Location	Approx Distance (ft)	Sampling Time (hrs)	Day of Illness	Nose VL on day of sample (copies/mL x 10 ⁴)
AH03†	Yes	Side room	3-7 & ≥7	1 & 3	4	17.3
AH04†	Yes	Side room	3-7 & ≥7	1 & 2	3	825
AH20	No	ITU main area	3 - 7	3	5	39.8
AH20	No	ITU main area	3 - 7	3	6	44.8
AH20	No	ITU main area	3 - 7	3	7	ND
AH20	No	ITU main area	3 - 7	3	8	3.3
AH27†	No	Side room	≥7	3	4	417
AH32†	No	Side room	≥7	3	4	5.5
AC20	No	Lounge	≥7*	3	4	340
AC26	No	Lounge	≥7*	3	5	2.0
AC28†	No	Lounge	≥7*	3	4	774
AC36†	Yes	Bedroom	≥7	3	4	0.4
CC05†	No	Bedroom	3-7 & ≥7*	1 & 3	3	57.9
CC15†#	Yes	Lounge	3-7 & ≥7*	1 & 3	3	2452
CH03†#	Yes	Shared room	3-7 & ≥7	3	5	1848

Footnote: Positive samples obtained, † = Rapid antigen test positive, # = other infected people present at the time of sampling, * = subject ambulatory at time of sampling, ND = Not Done

In Year 1 collections took place at different distances (3-7 and ≥7ft) from the subject and for different periods of time (1, 2 or 3 hours). In Year 2 only one collection was made per sampling episode; sampling took place over 3 hours and the sampler was positioned in a convenient location in the same room as the subject (usually this meant that the sampler was ≥7ft from the subject).

The following collections were made;

- A 12 samples collected at 3-7ft over 1 hour; five positive = 42%
- B 23 samples collected at 3-7ft over 3 hours; eight positive = 35%
- C 12 samples collected at ≥ 7 ft over 1 hour; three positive = 25%
- D 30 samples collected at ≥ 7 ft over 3 hours; seven positive = 23%
- E 3 samples collected at ≥ 7 ft over 2 hours; zero positive = 0%

Statistical analyses were performed to investigate any associations between the sampling parameters and a positive PCR result;

- A vs. B: Point estimates suggest that samples collected at a distance of 3-7ft were 34% more likely to be positive when the sampling duration was 1 hour, compared to an extended sampling duration of 3 hours. This result is not statistically significant. Unadjusted OR: 1.34 (95%CI: 0.25- 6.93); $p= 0.6891$.
- C vs. D: Samples collected at a distance ≥ 7 ft were approximately 10% more likely to be positive when the sampling duration was 1 hour as compared to an extended sampling duration of 3 hours. This result is not statistically significant. Unadjusted OR: 1.10 (95% CI: 0.15- 6.27); $p= 0.9088$.
- A vs. C: Samples collected over a 1 hour period were approximately twice as likely to be positive when they were collected at a distance of 3-7ft as compared to >7 ft. This result is not statistically significant. Unadjusted OR: 2.14 (95% CI: 0.28- 18.31); $p= 0.3865$.
- B vs. D: Samples collected over a 3 hour period were approximately 75% more likely to be positive when they were collected at a distance of 3-7ft as compared to >7 ft. This result is not statistically significant. Unadjusted OR: 1.75 (95% CI: 0.44- 6.96); $p= 0.3591$.

Virus was detected in all particle sizes collected; particles <1um gave 7 out of 26 positives (27%); 1-4um gave 9 out of 27 positives (33%) and >4um gave 7 out of 27 positives (26%).

Table 4.17: Positive air sample results

	AH03			AH04				CC15				CH03		AC36
Subject setting (+ infected others)	Hospital bed in side room			Hospital bed in side room				Playing in living room (6 year old infected child also present)				Cot on neonatal unit (2 infected neonates also present)		Bedroom
Room Temperature (°C)	21.6			23.3				18.0				24.0		17.0
Room Humidity (relative %)	50			50				60				40		44
Duration of sampling (hours)	1	3		1		2		1		3		3		3
Approximate distance from subject (ft)	3-7	≥7	3-7	≥7	3-7	≥7	≥7	3-7	≥7	3-7	≥7	3-7	≥7	≥7
Particle size detected in (µm) PCR copies/mL	<1 1068		<1 238 1-4 258 >4 511		1-4 603			<1 13199 1-4 5179 >4 8210	<1 5156 1-4 7107 >4 4028	N/A 1-4 24231 >4 5603	<1 2149 1-4 5166 >4 4889	<1 2577 1-4 3527 >4 3639	<1 1287 1-4 3889 >4 2245	1-4 5388

There were no differences in the GM viral loads on illness Day 4 or in LRT and URT scores on illness Days 3 and 4 between those with positive and negative air samples (Table 4.18).

Table 4.18: Viral loads and symptom scores compared between those with positive and those with negative air samples.

Illness day	GM nasal VL air positive (95% CI)	GM nasal VL air negative (95% CI)	GM ratio* (95% CI)	P value
Day 4	120.9 (1.1, 12902.5)	42.1 (7.9, 225.1)	2.9 (0.1, 140.8)	0.565
Illness day	Mean URT score air positive	Mean URT score air negative	Mean difference	P value
Day 3	6.3	7.0	0.75	0.84
Day 4	4.6	7.1	2.5	0.34
Illness day	Mean LRT score air positive	Mean LRT score air negative	Mean difference	P value
Day 3	3.8	3.0	-0.8	0.60
Day 4	2.8	4.1	1.3	0.28

*Footnote: * Reference category = air negative samples, GM = Geometric Mean, VL = Viral Load, URT = Upper Respiratory Tract, LRT = Lower Respiratory Tract*

4.4.9 Environmental sampling data summary

- 5% of swabbed surfaces were contaminated with influenza; viable virus was detected on two occasions.
- Positive surface samples were significantly associated with higher nasal viral loads on Day 3 and more upper respiratory tract symptoms.
- PCR positive air samples were obtained from 5 subjects. These samples were equally well represented across all particle size ranges collected, which includes respirable particles. We were unable to demonstrate the presence of viable virus in air samples.

4.4.10 Influenza ward

Surface swabs and air samples were collected over a 2 day period from a hospital ward in Sheffield that cohorted patients with influenza. Swabs were taken from amongst others things; door handle, desk, tap, medicine trolley, computer and telephone. On each day, four air samplers were positioned around the ward and collected specimens over a 3 hour period. On Day 1, ten patients with influenza A and five with influenza B were present. On average patients had been unwell for 12 days and all were either taking or had completed a course of oseltamivir. 22 swabs and 12 air samples were collected, all were negative by PCR. On Day 2, seven patients with influenza A and five with influenza B remained. A further 14 swabs and 12 air specimens were taken; all were again negative by PCR.

4.4.11 Influenza B

Data was collected from five subjects (four children and one adult) with influenza B, though follow up of these cases was short (mean = 2.4 days). Two of the cases (both children) were culture positive from nose swabs. Only one case (a hospitalised adult) received oseltamivir. 32 surface swabs were taken and tested, none were positive for influenza B by PCR.

4.5 Discussion

This is the first study to examine the relationship between influenza viral shedding from the nose with viral deposition in the near patient environment. In addition, it is the first time that the air around specific individuals with influenza has been sampled and examined for the presence of airborne virus.

4.5.1 Symptoms

Subjects' symptoms were diverse with the commonest being cough and sore throat. Symptoms peaked early in the course of illness in the majority of cases which is consistent with other reports (Carrat *et al*, 2008; Writing Committee of the WHO Consultation on Clinical Aspects of Pandemic Influenza 2010). Scores for all categories of symptoms were lower in children compared to adults on illness Days 3 and 4. This may be explained by reporting bias i.e. differences in reporting behaviour between adults and children, and the fact that parents usually reported symptoms of behalf of younger children.

4.5.2 Viral shedding

The mean duration of shedding detected by PCR was between 6-7 days and by culture was between 3-5 days. A variety of estimates were calculated in an attempt to overcome some of the limitations in sampling and testing and they should be interpreted carefully. No significant differences were observed between adults and children despite the widely held view that children shed virus for longer than adults (Frank *et al*, 1981; Sato *et al*, 2005). Reasons for this could include lack of statistical power to detect a difference and heterogeneity in the quality of sample obtained (this may be particularly relevant when dealing with children

when operator skill and compliance of the subject are important). There was also no significant difference between hospitalised and community cases though there was a trend to longer shedding in hospitalised cases which accords with previous data (Leekha *et al*, 2007; Lee *et al*, 2009) and has infection control implications for healthcare institutions. Our findings on the duration of viral shedding are broadly in agreement with published findings concerning seasonal influenza (Boivin *et al*, 2000; Lau *et al*, 2010; Ng *et al*, 2010) and A(H1N1)pdm09 (Cao *et al*, 2009; van Doorn 2009; Ling *et al*, 2010; Suess *et al*, 2010; To *et al*, 2010; Waiboci *et al*, 2011) (Table 4.19). When comparing studies it should be borne in mind that differences in study populations (children vs. adults, hospital vs. community cases), sampling methods and the proportions of cases receiving antivirals (particularly whether they received them within 48 hours) may exist.

Despite general agreement about the mean duration of shedding, data do suggest that some individuals shed virus for longer periods. Our findings suggest that over 25% of cases remain potentially infectious for at least 5 days. Findings from Spain showed that 16 out of 64 (25%) of hospitalised cases are PCR positive 7 days after diagnosis (Giannella *et al*, 2011), a study of 70 cases in Singapore revealed that shedding (by PCR) occurred for more than 7 days in 37% of patients (Ling *et al*, 2010) and in a Canadian study of community patients 74% (PCR) and 19% (culture) were positive on Day 8 of illness (De Serres *et al*, 2010). Whilst PCR is almost certainly a more sensitive test than culture because it detects both viable and non-viable virus (Ruest *et al*, 2003), its interpretation is problematic because it is not possible to determine the presence of viable (transmissible) virus, it can only illustrate the potential for it to be present.

This issue often causes difficulty in a clinical setting in deciding whether hospital cases are infectious.

Table 4.19: Published studies describing shedding patterns from cases of A(H1N1)pdm09.

	UK (current study)	China (Cao <i>et al</i> , 2009)	Hong Kong (To <i>et al</i> , 2010)	Singapore (Ling <i>et al</i> , 2010)	Germany (Suess <i>et al</i> , 2010)	Kenya (Waiboci <i>et al</i> , 2011)
Setting	Hospital and Community	Hospital	Hospital	Hospital	Community	Community
Number of cases	42	421	22	70	15	106
Adults and Children	Yes	Yes	Yes	Adults	Yes	Yes
% who received oseltamivir within 48hours	32%	72.4%	95%	51%	40%	1.8%
Duration of viral shedding (PCR)	6.5 (mean)	6 (median)	4 (median)	6 (mean)	6.6 (mean)	8 (median)
Duration of viral shedding (Culture)	3-5 (mean) Range 0-8	- -	- Range 1-5	4 (mean) -	- -	3 (mean) Range 0-13
Risk factors for prolonged shedding	-	Age <14, male sex, delayed oseltamivir	Younger age	-	-	-

It is generally well established that viral titres in URT samples taken from adults decline steadily from symptom onset and are proportional to symptom severity (Hayden *et al*, 1998; Treanor *et al*, 2000; Lau *et al*, 2010; Ng *et al*, 2010). Our results show a decline in viral titre over time but correlations between symptoms and viral load were poor. Factors to account for this may include; small sample size, lack of data for analysis early in the course of illness (Days 1 and 2), variation in the quality of sample collected, method of sampling, differences in the collation of

symptom data and viral sub-type studied. Some authors have shown that hospitalised cases shed more virus than community cases (Lee *et al*, 2009). However, we did not find any consistent trends to suggest that there are differences between the viral loads of hospitalised adults or children and those of community cases in our cohort. Indeed, a relationship between viral load and severe disease is not always demonstrated (Giannella *et al*, 2011; Lee *et al*, 2011). There are data to show that hospitalised adults shed more virus than hospitalised children. This is contrary to evidence that shows children tend to have higher viral loads (Hall *et al*, 1979; Lee *et al*, 2011); again our small sample size limits interpretation.

Surprisingly, symptom scores were significantly higher on Days 3 and 4 in those with shorter durations (<6 days) of PCR shedding compared to longer (≥ 6 days). A similar effect was not seen when shedding was detected by culture. We attempted to investigate this further by doing sub-group analyses but the effect was lost as statistical power diminished. Confidence in this finding is limited by small sample size as heterogeneities in sampling, virus detection, symptom reporting and inter-individual shedding are not dealt with satisfactorily.

4.5.3 Case History

One subject who shed live virus up until Day 8 of illness will be considered further. She was a 34 year old of south Asian origin who had no co-morbidities. She spent one night in hospital on the first day of her illness and began taking oseltamivir on Day 2. Prominent symptoms early in her illness were fever, cough, sore throat and fatigue. The virus was sequenced across the HA gene during the period of time that it was shed and no changes were detected. In addition, no common oseltamivir

resistance mutations were detected. All other household family members subsequently developed symptoms of cough and fever; a 5 year old daughter became unwell on Day 4 of the mother's illness followed by a 2 year old son on Day 5 and her 30 year old husband on Day 6. Thus a high SAR in this family was associated with high levels and prolonged shedding of virus in the index case (despite treatment with oseltamivir). The index case could be considered a super-spreader of infection.

4.5.4 Surfaces

A role for surfaces in the transmission of influenza A appears widely accepted but limited data are available to directly support the potential of contact transmission of influenza. Influenza can survive and remain viable on surfaces long enough to permit transmission, however, the ability to survive does not necessarily equate to the ability to infect; sufficient virus (enough to constitute an infectious dose) must be transmitted to initiate infection.

Virus contamination of surfaces was found in over a third (38%) of subject locations. However, despite finding that viral shedding continued for over 6 days in most subjects, virus was infrequently isolated from surface swabs (5.4%). On two occasions live virus was recovered. On the whole, amounts of virus recovered from surfaces during the study were small (53% had a Ct value >35) and the proportion of PCR positive samples where 3 out of 3, 2 out of 3 and 1 out of 3 aliquots of the same sample were positive was 31%, 11% and 58% respectively. No specific cleaning instructions were given during the follow up of our subjects, so for example daily cleaning of hospital rooms would have continued, which may have contributed to the low positive swab rate.

GM nasal viral loads were significantly higher in those with positive surface swabs compared to those with negative surface swabs on illness Day 3 (but not Day 4). In addition, there was a positive (but non-significant) correlation between nasal and surface viral loads. Furthermore, symptom scores were generally higher (significantly so for URT scores) in those with positive surface swabs. These findings suggest that individuals who emit the most virus into the environment are likely to be responsible for the majority of transmissions that occur via the contact route. The amount of virus released by individuals is governed by a number of factors but viral load and symptoms are chief among them. This data therefore supports the concept that super-spreaders of influenza infection might exist.

Sponges were trialled in Year 1 as they provide a potential advantage over swabs in that larger surface areas can be sampled. A disadvantage is that any virus collected is diluted in a relatively large volume of preserving medium soaked into the sponge making subsequent detection more difficult. Of the 18 sponge samples processed one was positive, giving a positivity rate of 9.1% compared to 1.4% for cotton swabs. Further investigation of the use of sponges is warranted.

Both swabbing and laboratory processing were more selective in Year 2 as we attempted to target samples that had a greater chance of positivity; the swab positive rates (1.4 vs. 10.7%) reflect this. For example, 4 out of 9 chosen surfaces in Year 1 (bedside table, dining table, patient table and windowsill) were not items that could be picked up or grasped by the hand and in many instances they were made of wood, a material that does not support virus survival (Greatorex *et al*, 2011). New items in Year 2 included cup, light switch and computer and a synthetic fibre tipped swab was used instead of cotton.

Several studies have assessed and documented the presence of influenza virus on surfaces around infected individuals (Boone and Gerba 2005; Bright *et al*, 2010; Pappas *et al*, 2010; Killingley *et al*, 2012). Our findings contrast with those of Boone and Gerba who detected influenza virus on over 50% of all swabs taken from a number of surfaces in the home and in child care centres. Reasons for this difference may include the presence of children in the location studied (100% v 33% in the current study) and the likelihood that more than one individual contributed to virus deposition in Boone's study in contrast to the circumstances of the current study where only one individual was ill when the majority of swabs were taken. More recent data comes from a randomised trial to investigate hand hygiene and surface contamination that took place in Thailand during 2009/10. One hundred and ninety one households containing an influenza positive child were recruited and 2358 swabs were collected on either Day 1, 3 or 7; 38 (1.6%) were positive by PCR (a Ct threshold of <40 was used). No swabs were culture positive. Twenty four (12.6%) households had at least one surface positive by PCR; 17 in a control arm, seven in an intervention arm that consisted of hand hygiene programme (prevalence risk difference = 10.3%; P=0.048). Households in which the index case was <8 years old had a significantly higher prevalence of contamination. Interestingly, reduced surface contamination in the hand hygiene group did not lead to a reduction in the secondary attack rate (Simmerman *et al*, 2010; Suntarattiwong *et al*, 2011).

As exemplified by our own work, differences between studies may be influenced by;

- Patients involved; we might expect more virus to be deposited if multiple cases contribute or if children are over represented compared to adults.

- The timing of swabbing in relation to the time of deposition and to the duration of illness in a case.
- Swabbing and detection methods.
- Virus survival; there is evidence to suggest that some viral strains may be more robust than others (Tiwari *et al*, 2006; Greatorex *et al*, 2011).
- Surfaces swabbed can differ in how frequently they are touched and their ability to support virus survival.

Overall the data suggests that either swabbing and/or laboratory methods for virus detection are insensitive or that virus deposited by infected patients does not contaminate the vast majority of surfaces in high titre. It is likely that both explanations have a role to play. To understand the relationship between surface contamination and contact transmission consider the situation in a household where an ill child contaminates the environment. There are likely to be specific high risk contamination events where a high titre of virus, in association with a volume of respiratory mucous that aids survival, is deposited e.g. sneezing directly onto a remote control or touching a door handle with a heavily contaminated hand. Being able to identify and sample surfaces involved in such events in a timely manner may be critical. Choosing a small number of surfaces to swab once a day may simply not be sufficiently targeted to collect samples associated with high transmission risk. In laboratory settings, the efficiency of retrieving live virus from a cotton swab, used to collect virus immediately after its inoculation onto non-porous surfaces, was 83-97% in one study (Bean *et al*, 1982) and approximately 50% in another (Greatorex *et al*, 2011); they are likely to be lower in field settings. With regard to laboratory related processes, pre-analytical (e.g. storage and transport of samples) and analytical variables (e.g. specificity and sensitivity of diagnostic tests), have major impacts on the performance of

laboratory testing. It is known for example that influenza virus is lost and survival reduced during freeze thaw cycles (Greiff *et al*, 1954).

Methodological improvements to surface sampling might therefore include;

- A more intensive and targeted swabbing protocol could be employed; e.g. shadowing an infected case around the home or using CCTV to pick out specific events.
- Sampling early in the course of a subject's illness when viral shedding is highest.
- Determination of the best swab to use in terms of size, material and design (e.g. flocked v standard).
- Consideration of other methods/materials for sampling e.g. foam, sponges (Otter *et al*, 2009; Lewandowski *et al*, 2010).
- Elimination or reduction of freeze thaw cycles.
- Selection of the optimal sample transport medium and eluent.

4.5.5 Air

If influenza can transmit via aerosols then we would expect to be able to detect virus in such aerosols. Studies performed over 40 years ago showed that artificially aerosolised influenza could be recovered (by using infection in animals as a detection method) for up to 24 hours after release (Wells and Brown 1936; Loosli *et al*, 1943) and that aerosolised virus is able to infect humans (Alford *et al*, 1966). More recently influenza virus has been detected (by PCR) in air samples taken from medical facilities (Blachere *et al*, 2009; Lindsley *et al*, 2010a) and from the directly exhaled breath of infected patients (Fabian *et al*, 2008). For the first time, this study now demonstrates that samples of air collected from around infected subjects contain influenza virus. This is important because it is

the only method to date that has assessed the amount of virus (released by an individual) that is available for transmission in normal room air.

All particle sizes collected contained virus detectable by PCR, including the <1µm and 1-4µm fraction sizes which are aerosols of a respirable size, i.e. they can reach the distal airways of the respiratory tract (Hinds 1999). Sampling nearer to the subject led to the detection of more virus as one might expect (as the concentration of virus in air will normally be higher nearer the source), though analyses did not reveal any statistical significance because numbers were small. Nasal viral loads, symptoms and duration of sampling time were not associated with positive air samples.

In Year 1, 4 out of 5 (80%) subjects sampled were positive compared to 1 out of 7 (14%) in Year 2. A number of factors may explain this;

- Sampling was generally done later in the course of illness in Year 2 (mean = 4.1 days) compared to Year 1 (3.6 days).
- 3 out of 5 (40%) subjects in Year 1 were reliably sampled at 3-7ft (i.e. were not ambulatory), compared to only 1 out of 7 (14%) in Year 2.
- Sampling of AH20 took place in a large open area (intensive care unit) compared to other subjects who were sampled whilst in single rooms.
- Increased volumes of VTM were used in Year 2 (1500 µl) compared to Year 1 (750 µl) as problems with small sample volumes were experienced in Year 1. This will dilute virus and may make detection more difficult.
- Two subjects in Year 1 from whom positive samples were obtained were sampled in the presence of other known influenza A cases who may have contributed to the airborne viral load.

Attempts can be made to understand whether the PCR copy number found in the air samples could represent an infectious dose. The ratio of the TCID₅₀ to the number of virions (and therefore to the number of genome copies) for influenza A has been estimated by various authors at 1:100, 1:400, 1:650 (Lamb and Krug 2001; van Elden *et al*, 2001; Wei *et al*, 2007). If we take 1 TCID₅₀ to equal 400 genome copies/mL, then an infectious aerosol dose [calculated to be 0.6 to 3 TCID₅₀ (Alford *et al*, 1966)] would be 240 – 1200 copies/mL. Such amounts were commonly collected by the samplers operating at 3.5L/min. By way of comparison, an adult human typically inhales 6L/min. If the virus collected/respired is infectious, then the majority of positive samples collected during this study could contain infectious doses of influenza.

Unfortunately we have been unable to conclusively demonstrate the presence of live A(H1N1)pdm09 in any samples. Initial culture results indicated the presence of live virus in three samples from one subject (AH03) and PCR detected only pandemic H1N1 in the original samples. However, following amplification of the virus to permit further analysis, it appears that the sample became contaminated with a laboratory influenza strain. Detecting live virus in air samples is challenging and techniques are in development. Difficulties include virus fragility (especially its susceptibility to desiccation) and the fact that sufficient virus needs to be collected to enable culture because the amount and concentration of virus being sampled in air is much lower than that from nasal swabs. The use of VTM during sample collection (as opposed to its addition afterwards) to help preserve virus has been cited as a necessity (Fabian *et al*, 2009a). However, preparatory work did not reveal this to be an absolute requirement with the samplers used (see Chapter 3).

4.5.6 Study Limitations

There are a number of limitations to this study. Firstly, the number of cases recruited was well below target. Reasons for this include; a) The study began just prior to the beginning of the second wave of the pandemic in England, but the overall number of people infected during the second wave was well below what had been predicted (Bowcott 2009) and seroconversions during the first wave were far higher than expected (Miller *et al*, 2010). A mild illness, including a high asymptomatic infection rate (Miller *et al*, 2010) contributed to the recruitment difficulties; b) Enrolling subjects early in the course of their illness was challenging; a significant proportion of subjects approached were ineligible because symptoms had been present for too long. An attempt to overcome this problem involved the recruitment of community as well as hospitalised cases (when presentation is often delayed); c) Identifying subjects as having influenza as opposed to other acute respiratory infections (ARIs) was problematic. It has been shown that the standard definition of ILI cannot be relied upon to distinguish A(H1N1)pdm09 from other ARIs (Chan *et al*, 2010a; Nguyen-Van-Tam *et al*, 2010) and the low numbers of people with illness in the local population made the positive predictive value of even our modified definition of ILI low (55%). A near-patient rapid antigen test was used to help identify influenza cases but the sensitivity of the test (using a nasal swab) was low (40%). These findings concur with a number of other reports about the low sensitivity of these tests to detect pandemic H1N1 (CDC 2009b; Ginocchio *et al*, 2009; Vasoo *et al*, 2009). In Year 1 the capacity to generate PCR results on samples quickly enough to limit follow up of non-influenza cases did not exist; in Year 2 this capacity was built in and resulted in time and cost savings. The modest recruitment of cases limits the study in several ways including the

generalisability of our findings and an inability to adequately address the primary aim.

Secondly, the difficulty of recruiting subjects early in the course of their illness meant that data on the initial days of illness were not collected. The first few days of illness usually see peak symptoms and viral shedding (and by inference environmental deposition). Unfortunately most analyses could only be attempted on illness Days 3 and 4. It is the lack of this data that hampers the ability to address the main aims of the study most.

Thirdly, the comparison of symptom data between adults and children is imperfect. The data collection method was the same and whilst this allows some comparison, it is clear that its interpretation must be guarded, as responses to the symptom diary card in children and adults may well be different; for example comprehension of questions, perceptions of symptoms and assessment of severity. A specific problem arises when symptoms are estimated by parents on the behalf of younger children. For these reasons symptom questionnaires have been designed specifically for adults (Barrett *et al*, 2005) and children (Jacobs *et al*, 2000). A method to compare them however, does not exist.

Fourthly, the majority of subjects from whom air samples were obtained (including all those with positive samples) were positive on rapid antigen testing. This may have biased the group somewhat as a positive rapid antigen test has been associated with higher viral loads in nasal samples (CDC 2009b). However, as our intention was to prove whether viable virus deposition on surfaces or in the air could be detected, selection of these individuals was important.

Finally, no measurements or estimates of room air flow patterns or ventilation were made when collecting samples. Such parameters are likely to have an influence on the ability to detect virus in the air.

4.6 Conclusion

Detecting virus, particularly live virus in the environment is challenging; getting to the subject in time, executing optimal sampling whilst preserving virus viability and performing sensitive detection tests in the laboratory are all key factors and present logistical challenges. Despite limitations important observations have been made and new evidence to inform the debate on the role of both contact and aerosol routes of transmission is presented. Data suggests that contact transmission via surfaces may be less important than hitherto emphasised, especially given the low titres of virus recovered and the scarce finding of live virus which together do not support the widespread presence of infectious doses of virus. The detection of virus by PCR in air collected at close range to subjects, well within the contact distance of an attending healthcare worker, suggests that the theory of short range bioaerosol transmission advanced by Tellier (Tellier 2009) cannot be dismissed. Although based on limited data these findings are of sufficient importance to justify further efforts to reproduce them, including further attempts to detect live virus, as they have important potential implications for infection control strategies.

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Chapter 5:

**Use of a Human Influenza
Challenge Model to Assess
Person to Person
Transmission -
Proof-of-Concept Study**

5.1 Abstract

Background

Distinguishing the relative importance of the three potential modes of influenza transmission is critical for the development of infection control advice for healthcare settings, schools, workplaces, and homes. To answer questions about routes of influenza transmission and the effectiveness of interventions to reduce it, both animal models and community studies are being employed but the limitations of these approaches hamper the generation of clear evidence which can be translated into policy. Experimental challenge of humans with influenza offers an alternative approach with many potential benefits. The primary aim of this proof of concept study was to establish that experimentally induced influenza infection is transmissible between humans.

Methods

Healthy subjects (Donors) deemed sero-susceptible to influenza A/H3N2/Wisconsin/67/2005 were intranasally inoculated and when symptoms began, further sero-susceptible subjects (Recipients) were exposed to Donors during an 'Exposure Event'. Subjects were split into three exposure groups, each consisting of two Donors and five Recipients. Subjects were in close contact, e.g. playing games and eating meals together, for a total of 28 hours during a 2 day period. Serum samples were collected from all subjects before and after exposure to virus and were tested for antibody evidence of infection. In addition, respiratory samples for PCR and viral culture were collected from all subjects after exposure.

Results

Among 24 healthy adult subjects, nine were randomised to the 'Donor' group and 15 to the 'Recipient' group. Following inoculation 5 out of 9 Donors (55%) developed illness and 7 out of 9 (78%) were proven to be infected (7 PCR, 4 culture and 7 serology); one Donor was found to have been non sero-susceptible at the time of inoculation. After exposure, 5 out of 15 Recipients developed symptoms and 3 out of 15 were proven to be infected (2 PCR, 0 culture and 1 serology). Three others were found to be non sero-susceptible prior to exposure. The overall attack rate in Recipients was 20% but was 25% after adjustment for pre-exposure immunity. There were no adverse events.

Conclusions

The model successfully demonstrated that transmission of influenza infection can be achieved from experimentally infected subjects. We had sought to generate a secondary attack rate of at least 25% in order that future studies could be run efficiently and this was achieved after adjustment for pre-existing immunity. Improvements to the study design could increase transmission rates further. Experimental human exposure studies remain an attractive method to answer challenging questions relating to influenza transmission.

5.2 Introduction

A better understanding of influenza transmission is needed to improve infection control and other disease mitigation strategies. A number of approaches to generate new insights into transmission are possible. Outbreak investigations can retrospectively provide information about transmission dynamics (e.g. reproductive number) but the uncontrolled nature of outbreaks themselves and the retrospective nature of data collection can make it difficult to reliably draw conclusions about the determinants of transmission, especially with regard to which routes of transmission were acting. Prospective intervention studies have the potential to address questions about routes of transmission but studies done to date have not provided conclusive findings (see Chapter 2, pages 52-59).

When investigating routes of transmission, there are advantages and disadvantages to conducting studies amongst naturally infected patients in the community. The prime advantage is that 'natural' infection transmission is studied. The main drawbacks concern timing, sample size, origin of infection and compliance.

- **Timing:** Infectiousness is likely related to viral shedding and symptoms and can therefore be expected to occur relatively early in the course of illness. It is therefore crucial that index cases and their contacts are identified early to enable interventions to be deployed before transmission occurs. Studies that rely on the presentation of infected individuals to medical services for recruitment will inevitably face delays. Further delay is caused by the need to recruit and educate household members and issue any intervention equipment. Studies have been conducted following the recruitment of subjects in advance

of an influenza season e.g. Flu Watch (Hayward 2011). Such a method could minimise delays to intervention but would require a substantial sample size (see below).

- **Sample size:** Attack rates of confirmed influenza and ILI observed during intervention studies conducted to date have been low which has meant that studies have been underpowered to detect differences in attack rates between study arms. Predicting influenza attack rates amongst susceptible persons is difficult and in the years prior to the 2009 pandemic (when many of the intervention studies were taking place) attack rates had generally been low both in the UK and US (HPA 2009; CDC 2011). In addition the assumed modest effect size of the interventions also places a requirement for a larger sample size. Thus there are significant logistic and financial barriers to recruiting the necessary numbers of volunteers to adequately power intervention studies.
- **Origin of infection:** We would like to be certain that any infections that occur in subjects are the result of contact with defined index cases, i.e. those around whom interventions are taking place. When a subject in a household study leaves the home or when a healthcare worker recruited to face mask study leaves their place of work they can become infected outside of the defined study setting. This may severely compromise the ability of the study to demonstrate the effectiveness of an intervention even if one existed.
- **Compliance:** To be effective intervention strategies must be adhered to. However, when interventions require specific behaviour modification outside of a normal routine we may expect less than 100% compliance. To better understand compliance with interventions such as hand hygiene and the wearing of face masks a number of factors must be considered; i) time spent performing an intervention,

ii) can steps be taken to reduce the impact of acceptable non-compliance e.g. removing a face mask at meal times and whilst sleeping, iii) adverse events related to an intervention, e.g. skin problems caused by hand hygiene, breathing problems related to face masks and iv) availability of equipment, e.g. hand hygiene gel, face masks. People are likely to be more compliant with an intervention when the perceived benefit outweighs any problems. In the case of influenza this might mean that people are more compliant during a pandemic or an outbreak that shows high morbidity and mortality. Incorporating these behaviour modifying factors into studies is difficult. Compliance rates with interventions in some studies conducted have been low; 21% reported good compliance with a face mask in one study (MacIntyre *et al*, 2009) whilst in another 26% reported regular use of a face mask and 55% reported good hand hygiene practice (which was only 9% more than the control group) (Cowling *et al*, 2009a).

These limitations of community intervention studies led to the consideration of alternative study methods and influenza challenge studies emerged as a prospect.

Experimental human challenge studies present an attractive way to study infectious diseases (assuming it is ethically acceptable to do so). Many aspects of the course of microbial disease (pathophysiology, transmission, treatments) can be observed in a manner seldom afforded by natural infection. There is a long history of challenge studies dating back to 1796 when Edward Jenner inoculated subjects with cowpox to protect them from smallpox (Riedel 2005). More recent studies include assessments of immunological correlates of protection against pneumococcal colonisation

(McCool *et al*, 2002), the evaluation of anti-malarial drugs and vaccines following malarial challenge (Epstein *et al*, 2007) and the evaluation of cholera vaccines (Tacket *et al*, 1999; Garcia *et al*, 2005). Challenge studies have been used to study viral respiratory infections since the 1940's. The MRC Common Cold Unit based in the UK conducted research involving approximately 18,000 volunteers between 1946 and 1989. Studies into the pathophysiology of infection, the spread of virus from person to person and the development and testing of vaccines and antiviral agents were performed (Tyrrell 1992).

Studies of respiratory virus transmission have revealed intriguing findings. Hall and Douglas concluded that transmission of respiratory syncytial virus (RSV) is predominantly mediated via close contact (through which both droplets and aerosols can act) and fomites. They found that volunteers who either cared for infected children ('cuddlers') or touched contaminated surfaces around infected children ('touchers') acquired infection whereas volunteers who sat at a distance of >6 ft from an infected child and had no contact ('sitters') did not (Hall and Douglas 1981). Rhinovirus transmission has been studied extensively using challenge experiments and models to predictably achieve experimental human to human transmissions have been devised (Gwaltney *et al*, 1978; Meschievitz *et al*, 1984). Routes of infection are analysed by manipulating conditions, e.g. deliberately contaminating hands or fomites, use of restraining devices to prevent face touching (eliminating the indirect contact route) and use of distance allowing only aerosols to act (Gwaltney *et al*, 1978; Dick *et al*, 1987). Such methods have shown that transmission can occur through indirect contact (via hands and fomites) and large droplets and/or bioaerosols. However, a review of rhinovirus transmission found it difficult to draw firm conclusions about routes of

transmission because studies have not been able to isolate all the different transmission routes and study designs were often somewhat contrived, e.g. directing behaviours and creating events that may not reflect natural conditions and occurrences (Hendley and Gwaltney 1988). Couch et al conducted a series of challenge experiments involving coxsackievirus, adenovirus and rhinovirus and concluded that both contact and airborne transmissions likely occur (Couch *et al*, 1966).

The first successful influenza challenge study took place in 1936 when volunteers were infected with atomised suspensions of infected mouse lung (Smorodintseff 1937). Noteworthy findings from influenza challenge studies that are relevant to transmission include;

- The aerosol route of infection led to the development of fever more frequently than did nasal inoculation (Henle *et al*, 1946).
- Natural infections cause more fever and LRT symptoms than do infections induced by nasal inoculation (Little *et al*, 1979).
- The infectious dose required for aerosol inoculation is thought to be significantly less than that required for nasal inoculation (Alford *et al*, 1966; Couch *et al*, 1966; Hayden *et al*, 1996).

In present-day influenza challenge studies, susceptible healthy adults are selected by serum antibody levels and infected intranasally with a well-characterized pool of wild-type influenza virus (the aerosol route of inoculation is not used as there is a concern that infections induced in this way may be more severe). Under these conditions, the majority of subjects will be infected and develop a mild illness accompanied by recovery of virus from the nasopharynx. This model has been used to evaluate antiviral agents and influenza vaccines (Carrat *et al*, 2008).

The safety of subjects recruited into influenza challenge studies is paramount and over the years influenza challenge has been shown to be safe. An extensive review of the literature has revealed only one subject who developed a serious adverse event that temporally followed experimental challenge with influenza (Ison *et al*, 2005). In July 2000, during a study of experimentally induced influenza (influenza B/Yamagata/88) to assess the prophylactic efficacy of the oral NAI peramivir, one subject developed myocardial dysfunction and presumed myocarditis that temporally followed influenza challenge. The subject was a 21-year-old man with no previous history of cardiac abnormalities. He did have a history of retinoblastoma that had been successfully treated with systemic chemotherapy, including an anthracycline agent, at 2 years of age. The pre-study evaluation revealed normal physical examination findings and laboratory studies, with the exceptions of an electrocardiogram (ECG) that revealed T wave flattening in the aVF and T wave inversion in lead III and an elevated creatine kinase (CK) level of 505 IU/mL. A second test performed prior to viral inoculation revealed a CK level of 202 IU/mL. The subject was inoculated and received the study drug. He shed low titres of influenza B and manifested only mild nasal symptoms, sore throat and loss of appetite. On Day 4 of the study, an ECG revealed new T wave inversions in leads II, aVF, and V4-6. Findings of an ECG performed 15 days after infection showed that the new abnormalities described on Day 4 had resolved. At the completion of the study, the subject took a 2 week vacation to Indonesia, during which time he experienced upper respiratory infection symptoms for several days. Because of the aforementioned ECG changes, the subject was asked to return for further evaluation. Fifty one days after influenza infection, he underwent echocardiography, which revealed left ventricular enlargement with severely reduced systolic function globally and minimal mitral and

tricuspid regurgitation. Physical examination revealed 6cm jugular venous distension and a faint mitral regurgitation murmur. An extensive laboratory work-up failed to reveal a secondary cause of myocardial dysfunction. He was treated with lisinopril and allowed to gradually increase his exercise levels. Successive echocardiograms at 1 and 5 months later showed progressive improvement in left ventricle function; the final study showed that the ejection fraction level had returned to low normal. The subject never developed any cardiac symptoms.

To further evaluate cardiac findings during influenza, 30 healthy adults without known cardiovascular disease who presented to clinic ≤ 72 hours after onset of influenza symptoms and had a positive influenza antigen study underwent serial ECGs, cardiac enzyme measurements, and echocardiography. Abnormal ECG findings were noted in 53%, 33%, 27%, and 23% of patients on Days 1, 4, 11, and 28, respectively, but none of the findings were considered to be clinically significant. No patient had significant changes in ejection fraction or abnormal wall motions seen on echocardiography (Ison *et al*, 2005). Furthermore, a recent review (Carrat *et al*, 2008) identified 56 studies involving 1,280 healthy adults that were challenged with influenza. This meta-analysis cited no other serious adverse events associated with the influenza challenge studies that were reviewed.

To date the challenge model has not been used to study the transmission of influenza amongst volunteers though it does present an attractive method of doing so (Killingley *et al*, 2011a). Advantages include the abilities to select serologically susceptible subjects who can be infected at specific times and control subject behaviour and environmental conditions. Studies could assess the efficacy of interventions such as face masks and

hand hygiene, and answer questions about virus survival in the environment, the effects of temperature and humidity, and the efficacy of engineering controls—e.g. ventilation and UV light. The generation of such information is fundamental to better understanding of routes of transmission and their relative importance. Furthermore, study of both pre-symptomatic and asymptomatic influenza infection and on the relationship between transmission and presence or level of symptoms should be possible.

Three approaches are possible to study transmission using a human challenge model: The first approach exposes volunteers to naturally infected patients. Although ideal because of natural infection, the use of patients infected naturally with influenza would be logistically complex (with a need to have immunologically naive volunteers ready and waiting), would raise ethical issues in relation to participants' safety, and could propagate a nosocomial outbreak. Such a study has been done to investigate transmission of RSV (Hall and Douglas 1981) but the safety concerns about adults infected with influenza are different to those with RSV.

The second approach sees participants undergo artificial challenge (not human to human) with particles or inocula of known size. Such studies have contributed to the evidence base on rhinovirus transmission (Hendley *et al*, 1973) and influenza transmission by demonstrating the potency of aerosol inoculation compared with instillation of nasal drops (Alford *et al*, 1966; Couch *et al*, 1966; Hayden *et al*, 1996). However, establishing the viral dose and composition (e.g. particle size) of inocula to mirror those that occur naturally is very difficult. Furthermore, virus shedding is a dynamic process dependent on the host and the environment; replication

of this process would also be difficult. Moreover, artificial transmission removes the important role that human-to-human interactions (physical and social) have in transmission.

In the third approach, volunteers are exposed to other, deliberately infected, volunteers. Two groups of volunteers are needed; 1) Donors are inoculated intranasally with virus and develop illness, 2) Recipients are exposed to symptomatic Donors. Experiments performed to study the transmission of a number of respiratory viruses have been done in the past (Couch *et al*, 1966; Gwaltney and Hendley 1982; Dick *et al*, 1987) but such a model has not been used to study influenza.

The third approach was considered a viable option and a proof of concept study was conducted to assess whether transmission of influenza infection can take place in a human challenge model.

5.3 Methods

The study took place between May and July 2009, and was conducted in accordance with the principles of the Declaration of Helsinki and UK regulatory requirements. It was approved by Plymouth Independent Ethics Committee (Retro 1236).

5.3.1 Objectives

The primary objective was to determine if an influenza A/H3N2/Wisconsin/67/2005 (A/WI) infection, induced by means of viral challenge, was transmissible between humans. Secondary objectives were to confirm the safety of transmitted infection, to determine the suitability

of the model for future trials and to obtain samples from the environment (surfaces and air) to look for the presence of influenza virus.

5.3.2 Recruitment

Recruitment occurred from a group of individuals who had expressed interest in participating in challenge studies conducted by Retroscreen Virology Limited (RVL). RVL uses a range of methods to recruit volunteers including posters, flyers, student and other local newspapers, magazines, radio, Facebook and its own website (www.FluCamp.com). RVL is also approached by volunteers who have heard of the studies through word of mouth. Individuals were contacted and asked if they would consider taking part in this study. Those that expressed interest were invited to attend for screening.

5.3.3 Screening

Volunteers needed to be healthy with no uncontrolled acute or chronic medical condition and be between the ages of 18-45. Two screening stages were used. The first was used to establish antibody susceptibility to the challenge virus and took place between study Days -200 and -14. The following were performed;

- Written, witnessed informed consent was obtained.
- A brief medical history was obtained to ensure criteria for enrolment were met.
- Approximately 10 mL of blood was obtained for influenza serum antibody (HAI) screening.

The second screening stage took place between study Days -45 and -5 and assessed volunteers against further study specific inclusion and

exclusion criteria (Table 5.1A+B). The following were performed (See Appendix 5.1 for a full schedule of assessments);

- A medical history was taken.
- A detailed explanation of the study was given to subjects remaining eligible (Appendix 5.2) and written informed consent (Appendix 5.3) to trial participation was obtained.
- Vital signs and baseline physical examination.
- An electrocardiogram (ECG).
- Nasal wash and throat swabs for compliance testing.
- Screening for class A drugs, alcohol and nicotine.
- Approximately 35 mL of blood was obtained for clinical haematology, coagulation and biochemistry safety assessments.
- HIV, Hepatitis B and Hepatitis C screening.
- Dipstick urine test.
- A urine pregnancy test.

Blood samples from subjects were also collected immediately prior to quarantine entry on Day -2 for repeat antibody testing (though results were not available until after the study). An HAI titre of ≤ 10 and/or an MN titre of < 80 were taken to indicate susceptibility to infection.

Table 5.1A: Inclusion criteria.

Inclusion Criteria
<ol style="list-style-type: none">1. Age 18 to 45 years, inclusive.2. Comprehension of the study requirements; availability for the required study period, ability to attend scheduled study visits, and willingness to participate in the inpatient challenge.3. Willingness to provide written consent for participation after reading the Participant Information Sheet and Informed Consent Form and after having adequate opportunity to discuss the study with an Investigator or qualified deputy.4. H3N2 HAI titre levels recorded as < 1:105. Good general health status as determined by a screening evaluation no greater than 160 days prior to the quarantine challenge phase.6. Subjects shall be registered with a general practitioner who will confirm a subjects' past medical history and their suitability to participate based on this. Consent will be obtained to receive this information.7. For female subjects, provision of a history of reliable contraceptive practices [hysterectomy or bilateral tubal ligation, oral or implanted contraceptive use, intrauterine device, barrier method plus spermicide, history of a single male partner with vasectomy and documentary evidence confirming sterility (negative sperm counts at the recommended post-operative intervals) or a history of abstinence deemed credible by the Investigator]. The provision of this history does NOT replace the requirement to perform, and obtain negative results in, pregnancy tests.

Table 5.1B: Exclusion criteria.

Exclusion Criteria
<ol style="list-style-type: none"> 1. Presence of any significant acute or chronic, uncontrolled medical or psychiatric illness, that in the view of the Investigator is associated with increased risk of complications of respiratory viral illness [subjects with uncomplicated chronic diagnoses stable and treated for ≥ 3 months (e.g. mild hypertension well-controlled with medication may be enrolled) provided the condition and its therapy are not known to be associated with an immunocompromised state or increased risk of complications of respiratory viral illness]. 2. Health care workers (including doctors, nurses, medical students and allied healthcare professionals) anticipated to have patient contact within 2 weeks of viral challenge. Healthcare workers who volunteer should not work with patients until 14 days after challenge or until their symptoms are fully resolved (whichever is the longer). Health care workers who work in units housing severely immunocompromised patients (e.g. bone marrow transplant units) were excluded from the study. 3. Venous access deemed inadequate for the phlebotomy demands of the study. 4. Positive serologic tests for HIV, Hepatitis B surface antigen, or Hepatitis C antibody. 5. Evidence of drug abuse or a positive urine Class A drug or alcohol screen. 6. Female subjects, who are known to be pregnant or who have a positive urine β-HCG test prior to challenge. 7. Acute (within 7 days of challenge/exposure) or chronic use of any medication or other product (prescription or over-the-counter), for symptoms of rhinitis or nasal congestion or for any nasopharyngeal complaint, or use of any intranasal medication for any indication (this includes any corticosteroid or beta agonist containing nasal spray). 8. Any history during adulthood of asthma, chronic obstructive pulmonary disease or any other condition requiring bronchodilator therapy. A history of childhood asthma until and including the age of 12 is acceptable. 9. Smokers unwilling/unable to desist for the quarantine phase duration of the study and any smoker with a >10 pack year history of smoking. A nicotine test was done at study specific screening and must be negative prior to admission to the quarantine unit.

10. Subjects who have type I or type II Diabetes Mellitus.
11. Body Mass Index >30.
12. An Abnormal ECG deemed clinically relevant by the Investigator.
13. Any anatomic or neurologic abnormality impairing the gag reflex or conducive to aspiration, or history suggestive of such a problem or any abnormality significantly altering the anatomy of the nose or nasopharynx.
14. Receipt of systemic glucocorticoids (in a dose \geq 5 mg prednisone daily or equivalent) within 1 month, or any other cytotoxic or immunosuppressive drug within 6 months prior to challenge.
15. Receipt of any investigational drug within 6 months prior to challenge, or prior participation in a clinical trial of any Influenza vaccine, or any investigational vaccine or experimental Influenza viral challenge delivered directly to the respiratory tract within 1 year prior to challenge.
16. History of adverse reaction to neuraminidase inhibitors e.g. oseltamivir
17. Presence of any febrile illness or symptoms of upper viral respiratory infection:
 - a. On the day of challenge/exposure or between admission for Influenza challenge/exposure and administration of the challenge inocula (Donors) or exposure event (Recipients). Such subjects may be re-evaluated for enrolment in later studies after resolution of the illness;
 - b. Within 2 weeks prior to challenge or if challenge is set to occur during November, December, January, February, or March if there are any symptoms suggestive of viral respiratory infection occurring between screening and challenge.
18. History of epistaxis (nose bleeds) more than 1 episode a month.
19. Presence of household member or close contact (for an additional 2 weeks after discharge from the isolation facility) who is: (a) less than 3 years of age; (b) any person with any known immunodeficiency; (c) any person receiving immunosuppressant medications; (d) any person undergoing or soon to undergo cancer chemotherapy within 28 days of challenge; (e) any person who has diagnosed emphysema or COPD, is elderly residing in a nursing home, or with severe lung disease or medical condition including but not exclusive to the conditions listed in Appendix 7; or (f) any person who has received a transplant (bone marrow or solid organ).

20. Any laboratory test which is abnormal and which is deemed by the Investigator to be clinically significant. (This includes blood chemistry, haematology, cardiac iso-enzymes, or urinalysis).
21. Known IgA deficiency, immotile cilia syndrome, or Kartagener's syndrome.
22. History of seasonal hay fever or a seasonal allergic rhinitis (SAR), including the use of symptomatic prescription only medication and non prescription medication.
23. As a result of the medical interview, physical exam, or screening investigations, the Investigator considers the subject unfit for the study.
24. Those employed or immediate relatives of those employed at Retroscreen Virology Ltd or the study site.
25. Staff and students working directly in or for any of the Units in which the principal or a co-Investigator works.
26. Immediate relatives of any of the principal or co-Investigators.
27. Receipt of a northern hemisphere seasonal influenza vaccine in the 2006/07/08 winter seasons.
28. Receipt of any systemic cytotoxic chemotherapy agent at any time.

Viral Challenge Exclusion criteria

1. History of hypersensitivity to chicken eggs.

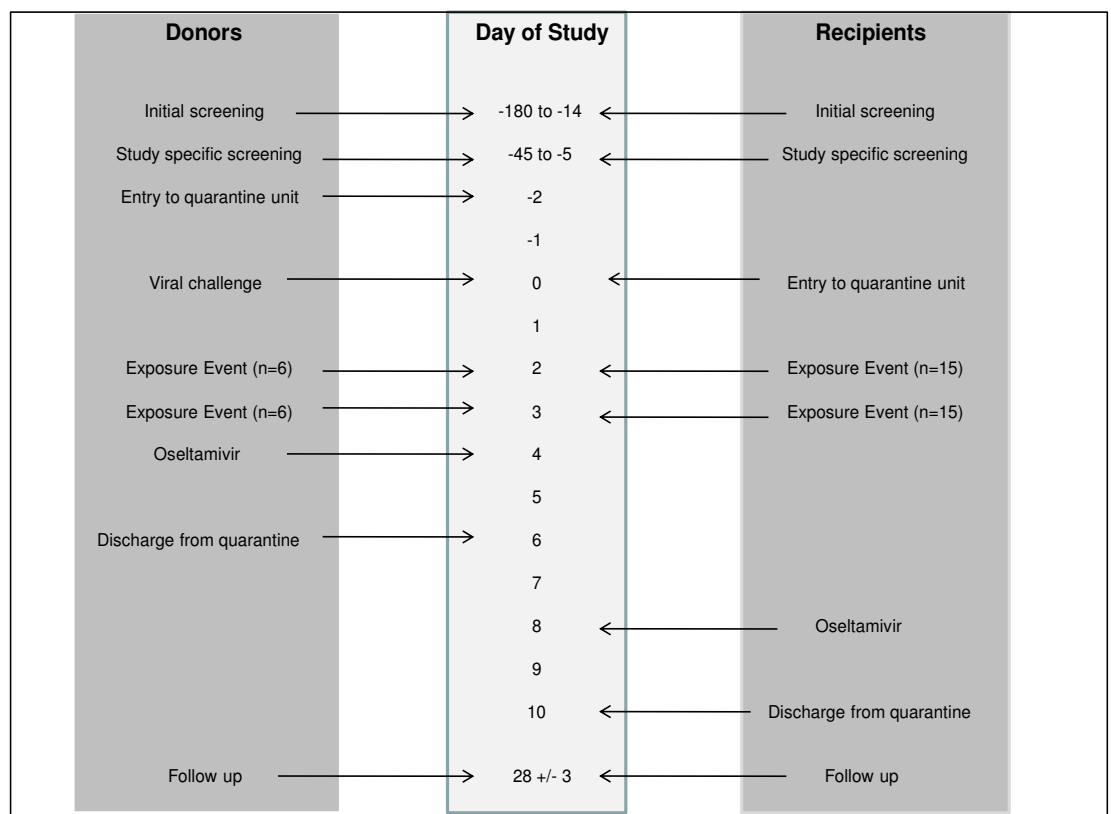
5.3.4 Study design and conduct

Sixty volunteers underwent stage two screening; 24 were eligible and were randomly allocated to one of two groups, 'Donors' or 'Recipients', prior to the start of the study (see Figure 5.1 for study timeline).

Ten Donors entered the quarantine unit on Day -2 and all remained well during 2 days of observation (Days -2 and -1). They were inoculated intranasally with A/WI virus on the morning of Day 0. Fourteen Recipients entered the quarantine unit on Day 0 and were segregated from Donors. In order to balance the groups in accordance with the protocol, one Donor

was randomly selected to become a Recipient (R12) on the morning of Day 0. Four Recipients reported a mild upper respiratory tract symptom during their period of observation (Day 0 to Day 1). One of these (R08) also reported diarrhoea on arrival at the quarantine site. He was monitored outside of quarantine until the morning of Day 2 by which time symptoms had resolved and he was allowed to enter the quarantine unit. Subjects were housed in pairs.

Figure 5.1: Study timeline.



On Day 2, 36 hours after inoculation, six Donors (selected on the basis of the highest symptom scores) and 15 Recipients were placed into shared accommodation and took part in three separate Exposure Events (EEs) each comprising of two Donors and five Recipients. The EE lasted for a total period of 30 hours and took place over study Days 2 (10.00 - 24.00)

and 3 (09.00 – 01.00). Subjects played games, watched television and ate meals together (Figure 5.2a, b & c) (Appendix 5.4). Donors and Recipients were separated overnight into shared rooms. The EE rooms measured 42.3m³ (4.6m x 4.0m x 2.3m) in size. Room temperature ranged between 22^oC and 26^oC and the humidity ranged between 38% and 53%. Windows were kept closed but recycling of air (not exchange) by an air conditioner allowed for subject comfort.

Follow up in the quarantine unit continued until Day 6 for Donors and Day 10 for Recipients. Recipients who developed symptoms were separated from roommates who did not have symptoms to prevent Recipient-Recipient transmission.

Clinical assessments and sample collections: (Appendix 5.1)

Subjects recorded symptoms of illness twice a day (08.00 and 20.00) during quarantine (Appendix 4.5). A record was kept of any concomitant medications (e.g. paracetamol) and adverse events. Vital signs were monitored four times a day and daily physical examination (Appendix 5.5), ECG and spirometry were performed. Venous blood was collected at specified intervals for serology and to examine a panel of safety parameters. Respiratory tract samples were obtained by nasal wash and throat swab and kept on wet ice for transport to the laboratory.

Influenza-like illness (ILI) was defined as an illness lasting 24 hours or more with either; i) fever >37.9 °C + at least one respiratory symptom or ii) two or more symptoms, at least one of which must be respiratory.

To minimise the possibility of post-experiment transmission, all subjects were given a 5 day course of oseltamivir (donors from Day 4 and recipients from Day 8) and had a negative rapid antigen test prior to

discharge. Analgesics and anti-pyretics were available at the discretion of the study physician.

End of study follow up for all subjects took place on Day 28 +/- 3. The following procedures were performed (Appendix 5.1);

- A blood sample for influenza serology was collected.
- ECG.
- A nasal wash and throat swab.
- Blood samples were collected for safety tests – haematology, coagulation and biochemistry tests (including cardiac iso-enzymes).
- Vital signs and physical examination.
- Adverse events were reviewed.
- New medical problems/diagnoses and any changes in concomitant medications were recorded.
- A dipstick urine test, including a pregnancy test for female subjects

Figure 5.2a: Subjects playing bingo during the exposure event.

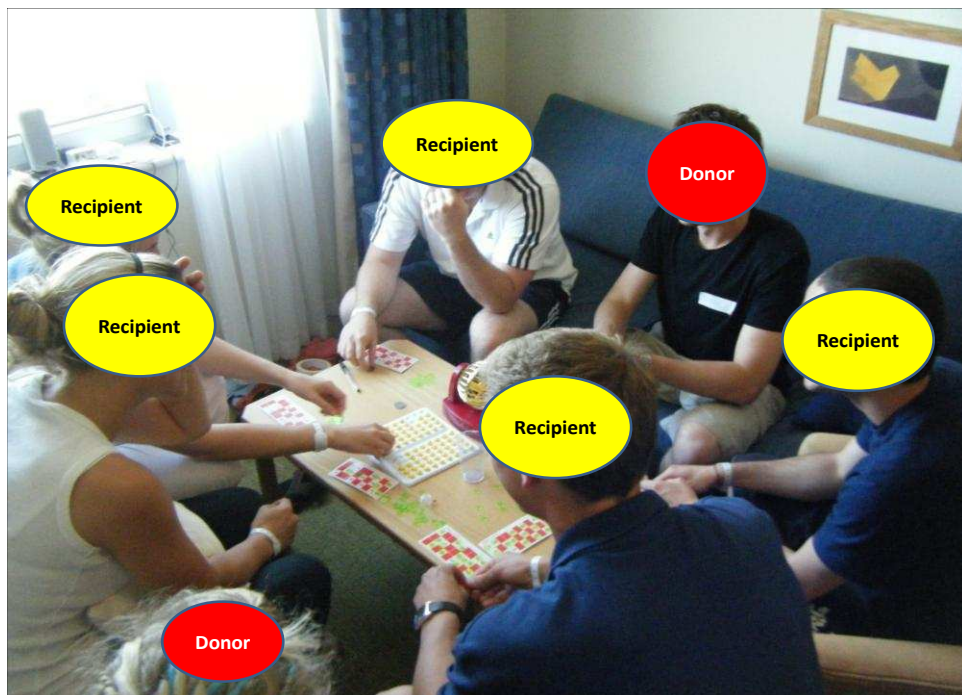


Figure 5.2b: Subjects playing cards during the exposure event.

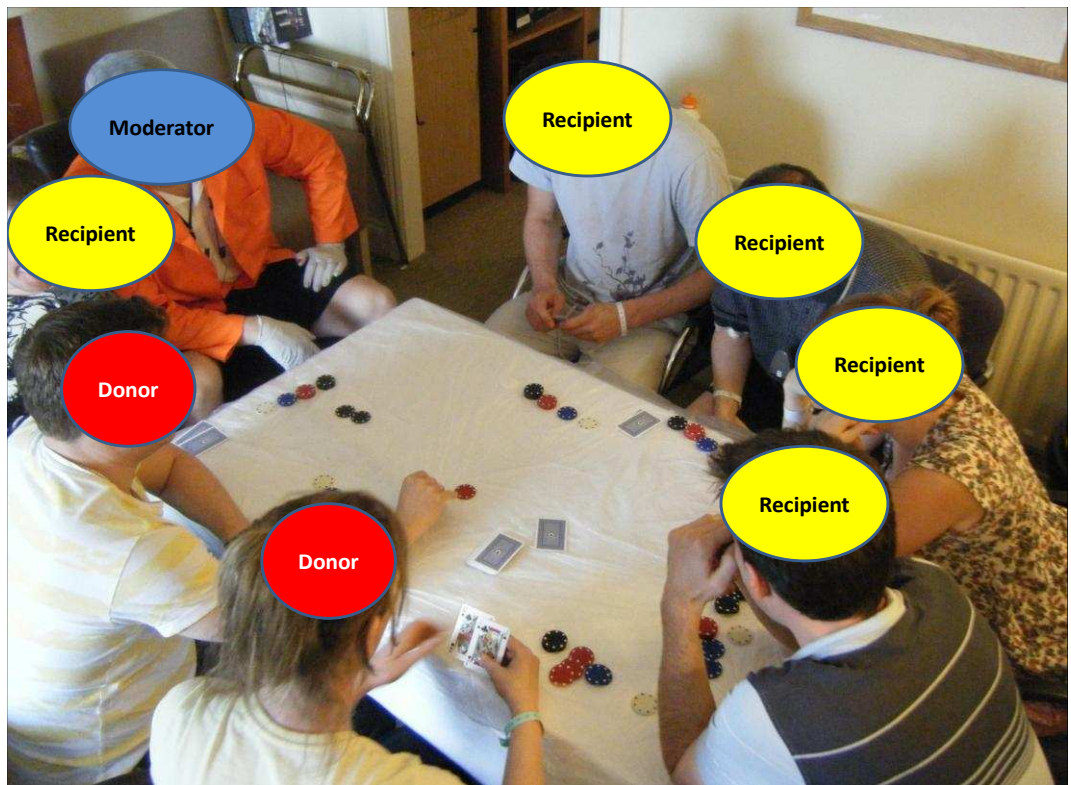
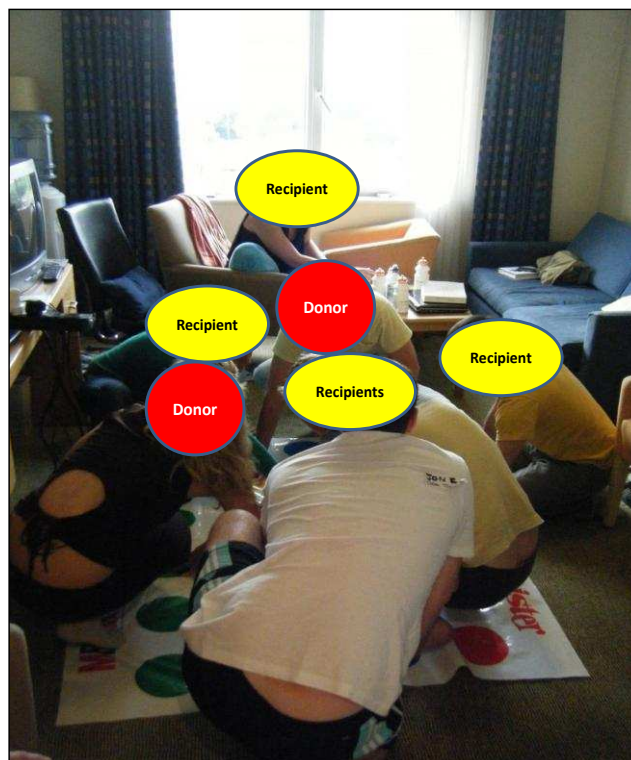


Figure 5.2c: Subjects playing Twister during the exposure event.



5.3.5 Challenge Virus

The challenge virus Influenza A/WI was used in this study. The viral stock was manufactured and processed under good manufacturing practices (GMP) and the final product underwent quality testing performed by the manufacturer (identity, appearance, sterility, infectivity, and contaminants) according to pre-determined specifications.

For inoculation, Donors were positioned supine with the chin up whilst a solution of 0.5 mL containing approximately $5.5 \log_{10} \text{TCID}_{50}/\text{mL}$ of virus was instilled into each nostril (0.25 mL per nostril twice). Subjects were instructed to remain in position for 10 minutes after inoculation and were then monitored by medical staff for a further 20 minutes in a seated position.

The challenge virus has been tested previously in both ferrets and humans:

- 12 ferrets were infected in a dose ranging study; no safety concerns were raised as a result of ferret inoculations (personal communication – RVL).
- 17 human subjects were quarantined and infected in a dose escalation study designed to assess the safety of inoculum and to establish the correct dosage of influenza virus to be used in future challenge experiments. No SAEs, unexpected symptoms or other complications occurred. Furthermore, no significant abnormalities were found on blood safety tests, ECG recordings or spirometry measurements. The data derived on symptoms that arose as a result of the two most optimal intranasal inoculation doses (n=8) showed that; 75% developed five or more symptoms, symptoms scores were highest approximately 60-90 hours post inoculation and viral shedding was

highest approximately 40-80 hours post inoculation (personal communication – RVL).

Comparisons between symptoms recorded following experimental challenge with H3N2 virus and natural infection (Monto *et al*, 2000; Kaji *et al*, 2003) are presented in Table 5.2

Table 5.2: Comparison of symptoms recorded during studies of natural and experimental infection.

Symptom	Monto n=2740 (majority H3N2)	Kaji n=98 (all H3N2)	Retroscreen n=17 (all H3N2- varying inoculated doses)	Retroscreen n=8 (all H3N2- optimal dosing range)
Fever	68%	94%	18%	38%
Malaise	94%	81%	29%	50%
Nasal congestion	91%	-	59%	100%
Cough	93%	18%	12%	25%
Sore throat	84%	38%	24%	25%
Sneezing	-	-	41%	75%

In consideration of the data presented in table 5.2, it is worth noting a few points;

- Subject selection; in the study done by Monto *et al*, subjects were recruited on the basis of fever or feverishness + two other ILI symptoms. Kaji *et al* recruited subjects who attended hospital. These studies may be selecting subjects at the upper end of the scale for symptom severity.

- Numbers; The RVL dataset is small compared to the others.
- Timing of symptom scores; Subjects were included by Kaji et al if they presented within 3 days of symptom onset, the paper by Monto et al does not state timelines. RVL data is presented as the maximum proportion observed between Days 2-4.

5.3.6 Environmental sampling

Swabs were taken from surfaces and commonly touched objects in subject bedrooms and EE rooms on study Days 2 and 3. Cotton swabs (FB57835; Fisherbrand) were moistened with VTM (VTM) and then rubbed across an area of 2x2cm² in six different directions applying even pressure.

Air samples were collected using a NIOSH two-stage cyclone bioaerosol sampler which has been described and validated for use with influenza (see Chapter 3). Three samplers were used during the EE on study Days 2 and 3; i) carried by a Donor, ii) carried by a Recipient and iii) free standing in the room at a height of 120cm. The samplers ran for 3 hours at a flow rate of 3.5 litres/min. Prior to sampling 750µl and 250µl of VTM were added to stage 1 and 2 tubes respectively. After sampling, the volume of VTM in both tubes was reconstituted to 750µl and the filter paper was immersed in a 15mL tube containing 750µl of VTM. Samples were placed on dry ice, transported to the laboratory and stored at -70°C.

5.3.7 Laboratory methods

Influenza serology at screening, quarantine entry (Donors = Day -2, Recipients = Day 0) and Day 28 was performed by HAI assay at RVL, London, UK). Serology was also performed on quarantine entry and Day 28 by HAI and MN assays at CDC laboratories, Atlanta, US. Culture of nasal wash and throat swab specimens was performed by RVL. Influenza

antigen rapid tests were performed on fresh nasal wash specimens using a Quidel QuickVue® Influenza A+B test. PCR analysis was performed at Lab 21 Healthcare laboratories, Cambridge, UK (influenza A) and HPA laboratories, Addenbrooke's Hospital, Cambridge, UK (influenza A and respiratory virus panel). Safety blood tests were performed by The Doctors Laboratory, London, UK. For further detail of laboratory methods see Appendix 5.6.

5.3.8 Outcome measures

A laboratory confirmed case was defined as evidence of acute infection based on four fold or greater rise in either HAI or MN titres between the Day -2 or Day 0 serum specimen and the Day 28 serum specimen, or a positive test by either viral culture or PCR. Any influenza case(s) amongst Recipients would be taken as evidence of infection and accepted as proof of concept that the virus is transmissible under experimental conditions. The PCR results presented are a combination of both HPA and Lab 21 results (see Appendix 5.7 for further detail). Serology results presented are those of CDC; RVL results were comparable (see Appendix 5.8).

5.3.9 Statistical methods

Sample size was based on the premise of attaining a SAR of $\geq 25\%$, defined in advance as the level of SAR that would be consistent with the viability of future transmission studies. Assuming a 25% attack rate and that the infection of each Recipient is independent of that of any other Recipient (i.e. no correlation of infection risk by EE group), using six Donors and 15 Recipients (in three groups of 2:5) gives a 76% chance of observing three or more cases. If no cases were detected, the upper 95% confidence bound on the attack rate would be 22%.

Descriptive statistics and reports are provided for subject demography, adverse events and responses to viral challenge and exposure. Summary statistics have been generated for signs and symptoms of influenza illness and viral assessments by PCR, culture and serology.

5.4 Results

Twenty four subjects (median age 27) satisfied the entry criteria and subsequently entered the quarantine unit.

5.4.1 Donor inoculation

Nine Donors (five male and four female) were intranasally inoculated on Day 0. The virus inoculum used to infect the donor subjects was back-titrated on MDCK cells at the time that the first subject was inoculated and against the time that the last volunteer was inoculated. Titres were found to be within acceptable ranges;

- The titre of the virus immediately post preparation was calculated to be $5.75 \log_{10}\text{TCID}_{50}/\text{mL}$.
- The titre of virus at the time that the first subject was inoculated was calculated to be $6.00 \log_{10}\text{TCID}_{50}/\text{mL}$.
- The titre of virus at the time that the last subject was inoculated was calculated to be $5.27 \log_{10}\text{TCID}_{50}/\text{mL}$.
- The GM of the virus titres at the time of the first and last subject inoculations was calculated to be $5.64 \log_{10}\text{TCID}_{50}/\text{mL}$.

5.4.2 Donor challenge

Seven out of 9 Donors were found to be infected (78%); four had symptoms consistent with an ILI [one (D05) had a recorded fever] and

three were asymptomatic. One of the two non infected Donors reported symptoms (Table 5.3 and Figure 5.3)

Table 5.3: Donor symptom scores.

Donor	Symptom Scores						
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
D1[#]	0	1	6	9.5	11	4.5	1.5
D2[#]	0	0	6.5	12.5	7.5	1.5	0
D3[#]	0	1	4	3.5	1	2	0
D4	0	0	0	0	0	0	0
D5[#]	0	0	0	9.5*	9*	4	0
D6	0	0	0	2	1.5	0	0
D7	0	0	0	0	0	0	0
D8	0	0	0	0	0	0	0
D9	0	0	0	0	0	0	0

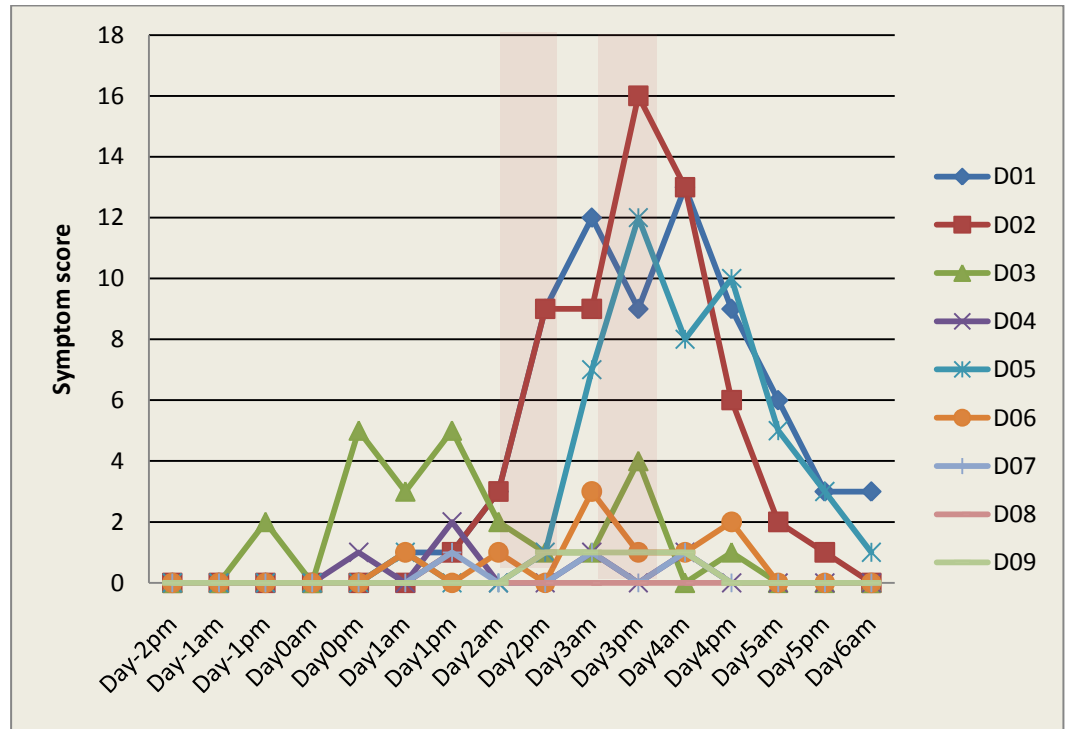
*Footnote: *Fever $\geq 38^{\circ}\text{C}$, [#]Donor with ILI
Scores shown are the average sum of the total symptom score for each day. Total symptom score is the sum of all individual symptom scores recorded at a particular time. A score of 0.5 has been recorded as 0 to show only more significant symptoms. Shading represents participation in the Exposure Event. D6 and D8 were not infected.*

5.4.3 Exposure Event

On Day 2, three Donors who showed symptoms consistent with ILI (D01, D02, and D03) and one who had respiratory symptoms but did not fulfil the definition of ILI (D06) were selected for the EE. In addition, two further asymptomatic Donors were randomly selected to take part (D04, D08). On Day 3, one of the three reserves (D05) became symptomatic

with an ILI. Out of the Donors who remained asymptomatic one (D08) was randomly withdrawn in favour of D05 (Table 5.3).

Figure 5.3: Graph showing Donor total symptom scores over time.



Footnote: The shaded areas represent the Exposure Event periods. Total symptom score is the sum of all individual symptom scores recorded at a particular time.

Four Donors were culture positive from nasal wash, seven were PCR positive on nasal wash (three were also positive on throat swab) and seven seroconverted (Table 5.4). One Donor (D04) was retrospectively found to have high serum antibody titres (HAI=160; MN=1280) on Day -2 and was therefore not sero-susceptible.

Viral shedding from the seven infected Donors was detected up to an average of 5.3 days following inoculation (this is a minimum value as four Donors shed virus up until the last day of testing). The average incubation

period for the four infected and symptomatic Donors was approximately 1.8 days. Viral shedding therefore occurred for an average of 3.5 days (5.3 – 1.8) after symptoms began in these four subjects.

Table 5.4: Results of Donor influenza challenge.

Subject	Results				Infection Status
Donor	ILI (day)	PCR	Culture (TCID50/mL)	Serology (Day -2 → 28)	
D01*	2-6	NW4-6	NW3 (3.5)	HAI 5 → 40 MN 28 → 640	ILI Infected
D02*	2-5	TS3 & NW4-6	NW4 (3.0)	HAI 5 → 20 MN 14 → 80	ILI Infected
D03*	2, 3, 5	NW3-4	-	HAI 40 → 80 MN 160 → >1280	ILI infected
D04*	-	-	-	HAI 160 → 160 MN >1280 → >1280	Immune pre-exposure
D05*	3-5	TS3 & NW4-6	NW3 & 4 (4.75 & 3.75)	HAI 5 → 80 MN 10 → 640	ILI Infected
D06*	-	-	-	HAI 5 → 5 MN 40 → 80	Symptomatic Not infected
D07	-	NW3-5	NW4 (2.0)	HAI 5 → 5 MN 10 → 40	Asymptomatic infected
D08*	-	NW 5-6	-	HAI 5 → 5 MN 20 → 80	Asymptomatic infected
D09	-	TS3 & NW4	-	HAI 10 → 80 MN 80 → >1280	Symptomatic infected

*Footnote: *Donor used in the exposure event. ILI = days when subject recorded symptoms consistent with ILI. Positive virological results are shown in red. Yellow shows pre-existing antibody immunity. Numbers refer to day(s) when positive e.g. NW4-6 = Nasal wash positive Day 4, 5 and 6. Serology results are presented as geometric mean titres. A positive result is a fourfold rise in titre. TS = Throat swab, HAI = Haemagglutination Inhibition, MN = Microneutralisation*

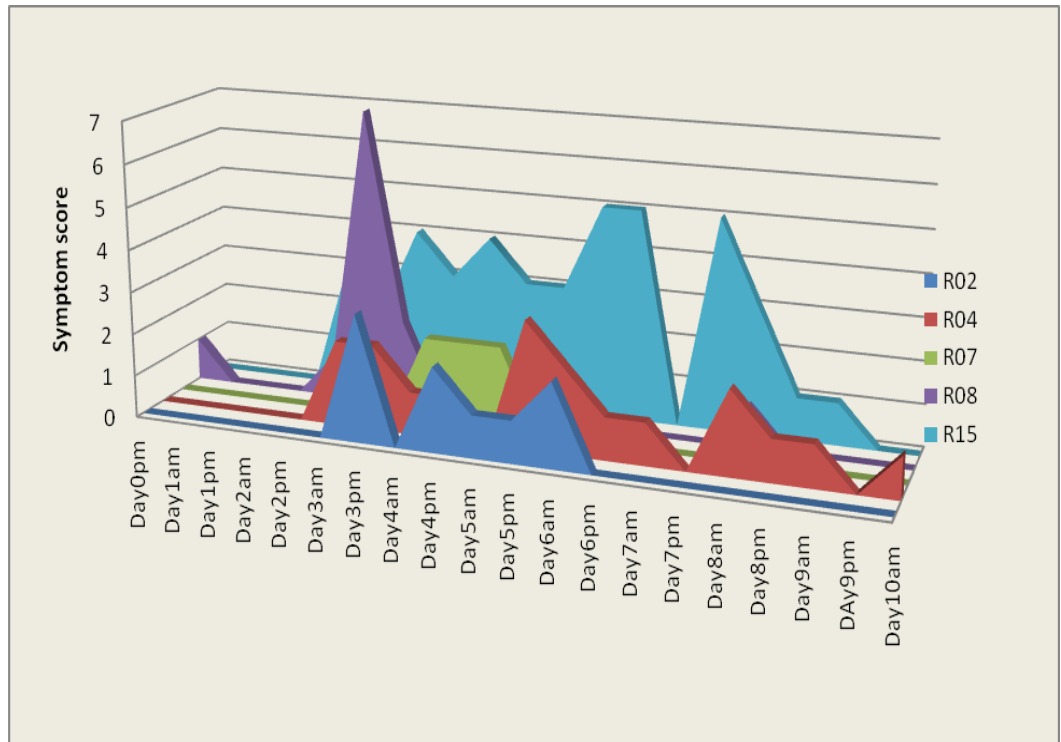
No clinically significant ECGs, spirometry or vital signs and no unexpected or clinically significant blood tests amongst Donors were recorded. One adverse event occurred; D05 suffered a nose bleed that resolved spontaneously on Day 6, this was deemed 'probably' related to influenza challenge. All Donors were rapid antigen test negative on discharge (Day

6) from the quarantine unit, completed a course of oseltamivir and remained well through the Day 28 follow-up visit.

5.4.4 Recipient exposure

Following the EE, ten Recipients reported symptoms, of whom four (R04, R07, R08 and R15) had illness consistent with ILI (Figure 5.4 and Table 5.5).

Figure 5.4: Graph showing selected Recipient symptom scores over time.



Footnote: Graph shows Recipients who demonstrated symptoms on at least two consecutive days

Table 5.5: Recipient symptom scores.

Recipient	Symptom Scores									
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	
R1	0	0	0	0	0	0	0	0	0	0
R2	0	0	0	1.5	1	1	1	0	0	0
R3	0	0	0	0	0	0	0			
R4	0	0	0	1.5	1	1.5	1.5	0	1.5	
R5	0	0	0	0	0	0	1.5	0	0	0
R6	0	0	0	0	0	0	0	0	0	0
R7	0	0	0	0	2	1	0	0	0	0
R8	0	0	0	4.5	0	0	0	0	0	0
R9	0	0	0	0	0	0	0	0	0	0
R10	0	0	0	0	0	0	0	0	0	0
R11	0	0	0	0	0	0	0	0	0	0
R12	0	0	1	0	0	0	0	0	0	0
R13	0	0	0	0	0	0	0	0	0	0
R14	0	0	0	0	0	0	0	0	0	0
R15	0	0	1.5	3	3.5	3	6	4	2	

Footnote: Scores shown are the average sum of the total symptom score for each day. Total symptom score is the sum of all individual symptom scores recorded at a particular time. A score of 0.5 has been recorded as 0 to show only more significant symptoms. Shading shows Recipients who had symptoms consistent with ILI for ≥ 24 hours.

Three (20%) Recipients (R08, R12 and R15) had laboratory confirmed influenza; R12 was confirmed serologically and R08 and R15 were PCR positive on a nasal wash from Day 4 and Day 6 respectively (Table 5.6). Three recipients had MN titres ≥ 80 on Day -2 and were considered non sero-susceptible.

Table 5.6: Results of Recipient influenza challenge.

Subject	Results				Influenza Infection Status
Recipient	ILI (day)	PCR	Culture	Serology (Day -2 → 28)	
R1	-	-	-	HAI 10 → 10 MN 80 → 80	Immune pre-exposure
R2	-	-	-	HAI 10 → 7 MN 57 → 57	Asymptomatic not infected
R3	-	-	-	HAI 5 → 5 MN 40 → 40	Asymptomatic not infected
R4[#]	5-6	-	-	HAI 5 → 5 MN 40 → 40	Asymptomatic not infected
R5	-	-	-	HAI 5 → 5 MN 20 → 10	Asymptomatic not infected
R6	-	-	-	HAI 5 → 5 MN 20 → 14	Asymptomatic not infected
R7	4-5	-	-	HAI 5 → 10 MN 80 → 160	Immune pre-exposure
R8	3	NW4	-	HAI 5 → 5 MN 20 → 10	ILI Infected
R9	-	-	-	HAI 10 → 5 MN 40 → 40	Asymptomatic not infected
R10	-	-	-	HAI 5 → 5 MN 20 → 40	Asymptomatic not infected
R11	-	-	-	HAI 5 → 5 MN 20 → 40	Asymptomatic not infected
R12	-	-	-	HAI 5 → 20 MN 40 → 160	Asymptomatic infected
R13	-	-	-	HAI 40 → 40 MN 160 → 160	Immune pre-exposure
R14	-	-	-	HAI 5 → 5 MN 20 → 20	Asymptomatic not infected
R15[#]	3-8	NW6	-	HAI 5 → 5 MN 20 → 10	ILI Infected

Footnote: # Rhinovirus detected, ILI = Days when subject recorded symptoms consistent with ILI. Positive virological results are shown in red. Yellow shows pre-existing antibody immunity. Serology results are presented as geometric mean titres. A positive result is a fourfold rise in titre. HAI = Haemagglutination, MN = Microneutralisation, NW = Nasal wash

No clinically significant ECGs, spirometry or vital signs and no unexpected or clinically significant blood tests amongst Recipients were recorded. All were rapid antigen test negative on discharge from the quarantine unit, completed a course of oseltamivir and remained well through the Day 28 follow-up visit.

5.4.5 Attack Rates

Attack rates in each of the three EE groups (A, B, C) were 0%, 20% and 40% respectively. Excluding non sero-susceptible Recipients gives attack rates of 0, 25% and 50% per group and an overall attack rate of 25% (95% CI 6-57%) (Table 5.7a-c).

Table 5.7a: Outcome of Exposure Event for Group 1.

Group 1 Subjects	Symptoms post virus exposure	ILI	Fever	Influenza status
D03	Yes	Yes	No	Infected
D04	No	No	No	Immune
R01	No	No	No	Immune
R02	Yes	No	No	Not Infected
R03	Yes	No	No	Not Infected
R04 [#]	Yes	Yes	No	Not Infected
R05	Yes	No	No	Not Infected
Attack rate = 0%				

Footnote: # Infected with rhinovirus

Table 5.7b: Outcome of Exposure Event for Group 2.

Group 2 Subjects	Symptoms post virus exposure	ILI	Fever	Influenza status
D01	Yes	Yes	No	Infected
D08*	No	No	No	Infected
D05†	Yes	Yes	Yes	Infected
R06	No	No	No	Not Infected
R07	Yes	Yes	No	Immune
R08	Yes	Yes	No	Infected
R09	No	No	No	Not Infected
R10	Yes	No	No	Not Infected
Attack rate = 20% (adjusted for immune Recipients = 25%)				

Footnote: * Took part on Day 2 only, † Took part on Day 3 only

Table 5.7c: Outcome of Exposure Event for Group 3.

Group 3 Subjects	Symptoms post virus exposure	ILI	Fever	Influenza status
D02	Yes	Yes	No	Infected
D06	Yes	No	No	Not Infected
R11	Yes	No	No	Not Infected
R12	No	No	No	Infected
R13	Yes	No	No	Immune
R14	No	No	No	Not Infected
R15#	Yes	Yes	No	Infected
Attack rate = 40% (adjusted for immune Recipients = 50%)				

Footnote: #Infected with influenza and Rhinovirus

5.4.6 Co-infections

A panel of respiratory virus PCRs performed on Recipient nasal washes from days 4-7 detected rhinovirus from R15 (Day 4) and R04 (Day 6). All Donor nasal washes on Day 3 were negative for non-influenza viruses.

5.4.7 Environmental sampling

Air sampling took place within EE group B on study Days 2 and 3 and generated 18 samples. Samplers were carried by D01 and R10 and one sampler was free standing. Only one sample was PCR positive; this was in 1-4 μm size range. This sample was collected on Day 3 by a device carried by a Donor (D01). Culture assays conducted on this and all other samples were negative.

Surface swabbing was performed in two subject bedrooms (D01 and D02/03) and the EE rooms of groups A and B on study Days 2 and 3. All rooms where sampling took place contained Donors who were proven to be infected with influenza. Forty eight samples were collected from the following surfaces; Bedside table (wood), computer game control (plastic), teaspoon handle (metal), door handle (metal), drinking glass, plastic water bottle, plastic dice, ceramic mug, computer keyboard, computer touchpad, paper cup, coffee table (wood), plastic table cloth. Nine out of 48 (19%) samples were PCR positive;

- Donor bedroom (D01) Day 2 – Ceramic mug and computer touchpad.
- Donor bedroom (D01) Day 3 – Computer touch pad.
- Donor bedroom (D02/03) Day 3 – Bedside table, teaspoon, door handle, ceramic mug, plastic bottle.
- Exposure room Day 3 – computer touchpad.

All samples were culture negative.

5.5 Discussion

This study has demonstrated for the first time, the successful and safe deployment of a human challenge model to demonstrate transmission of influenza infection from experimentally infected subjects to other susceptible subjects. The overall attack rate was 20%, but taking into account non sero-susceptible (immune) Recipients, the adjusted attack rate was 25%. In addition the presence of influenza (by PCR) on surfaces and in the air around subjects has been demonstrated. The human challenge model could offer opportunities to study the transmission of influenza and is an alternative to the study of naturally infected subjects.

In this study most sero-susceptible Donors (88%) developed infection which is in keeping with other challenge studies (Carrat *et al*, 2008). It is not clear why higher rates of infection are not achieved but probably relates to some degree of underlying heterosubtypic or T-cell mediated immunity. Five (63%) of the infected susceptible Donors developed symptoms which is similar to an overall rate (65%) observed in other H3N2 challenge studies, though less fever was observed (13% v 41%) (Carrat *et al*, 2008).

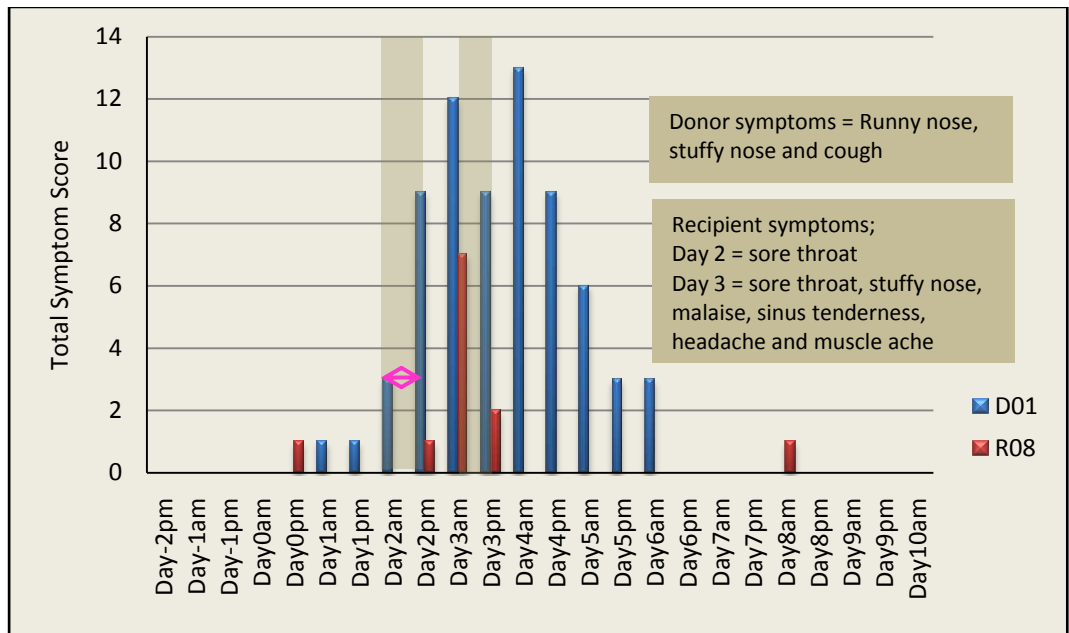
Although the majority of Recipients experienced symptoms post EE, most were not clinically significant and may relate to the effect of quarantine itself (e.g. nasal congestion due to confinement indoors). Three out of four Recipients who had an ILI, had virological evidence of infection (two influenza and one rhinovirus). Although we could confirm influenza infections in three Recipients, we recognise the lack of coherence across diagnostic modalities, particularly that antibody responses to the

transmitted infection were not strong. This likely reflects low viral loads and the mild nature of illness seen.

Antibody detection was undertaken by both HAI and MN assays. Virus neutralization assays are largely regarded as being a more sensitive method (Gross and Davis 1979) but there are, in general, no immune correlates of protection for neutralising antibody. Virus neutralization assays likely detects a broader range of antibody than does the HAI including antibody directed against the stem region of the HA molecule (Sui *et al*, 2009; Corti *et al*, 2010). Whilst a MN titre of 80 indicates some level of pre-existing antibody, it is not known whether this represents a protective level that would prevent infection. For Recipients R01 and R07 we have assumed that it did. However, two Donors (D09 and D03) had baseline MN titres of $\geq 1:80$ and were infected. It is possible that the total inoculation dose of approximately $5.64 \log_{10} \text{TCID}_{50}$ per Donor represents a substantially larger inoculum relative to the 'doses' to which the Recipients were exposed and that this larger amount of virus was able to overcome existing levels of MN antibody.

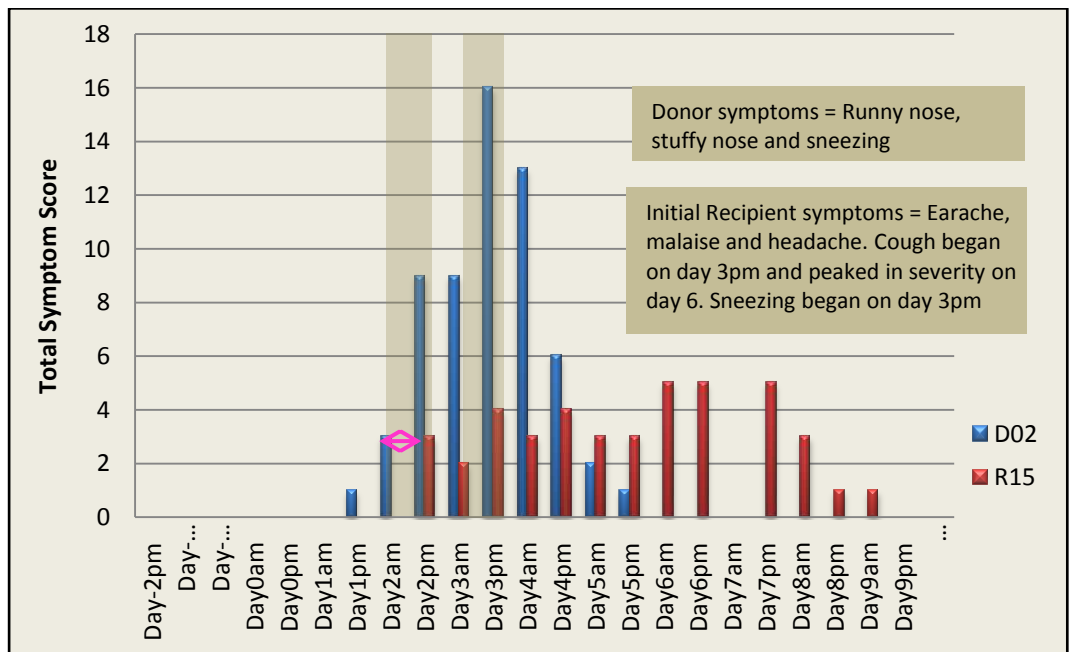
Based on the times that symptoms began, it appears that R08 caught and incubated infection from D01 within 12 hours. This illness transmission timeline is depicted in Figure 5.5a. The incubation period in the Recipient is more expedient than might have been expected but is plausible (Lessler *et al*, 2009). The finding of rhinovirus on a sample from R15 makes it difficult to interpret a similar timeline for transmission from D02 to R15 as rhinovirus may well have been causing the early symptoms seen in R15 (Figure 5.5b).

Figure 5.5a: Timelines of symptoms in Donor D01 and Recipient R08.



Footnote: The arrow indicates the 12 hours it took for illness to appear in R08. The shaded areas represent the Exposure Event periods. R08's symptom on Day 0 was earache. They also reported diarrhoea and the subject was isolated outside of the quarantine unit until these symptoms had resolved. am = 08.00, pm =20.00.

Figure 5.5b: Timelines of symptoms in Donor D02 and Recipient R15.



Footnote: The arrow indicates the 12 hours it took for illness to appear in R15. The shaded areas represent the Exposure Event periods. In R15, Rhinovirus was detected on Day 4 and influenza on Day 6. am = 08.00, pm =20.00.

The detection of rhinovirus (by PCR) in two Recipient subjects (one of whom was also infected with influenza during the EE) was unexpected. These two subjects did not share a room or take part in the same EE. Mild, single, upper respiratory tract symptoms were recorded by four other Recipient subjects prior to the EEs, so it is possible that they introduced rhinovirus into the quarantine unit. No staff members reported any illness. It is crucial for the integrity of challenge studies that steps are taken to prevent the introduction of other infections into a quarantine unit. A 2 day observation period was included in this study for this purpose, but in the future, consideration should be given to screening for a panel of respiratory viruses in both subjects and staff on entry to the quarantine unit.

Samples taken from the environment around infected subjects show that virus is deposited by experimentally infected individuals (swab positive rate 19%), validating the model for investigations into the role of contact transmission. The findings can be compared to other studies which have detected influenza virus (by PCR) on a number of surfaces in different settings with swab positive rates of 5.4% (Chapter 4 of this thesis), 3% (Simmerman *et al*, 2010) and >50% (Boone and Gerba 2005). Reasons for this variation may include study setting, presence of children and virus subtypes studied (see Chapter 4, page 144-145).

Evidence supporting the potential for bioaerosol transmission of influenza infection has been reviewed (Tellier 2009). Corroborative evidence includes the detection of influenza virus (by PCR) in the air around patients in respirable sized particles (Blachere *et al*, 2009; Lindsley *et al*, 2010a) and this study contributes new data. Detecting the presence of influenza in the environment is the first step in a chain of evidence needed

to confirm that influenza viruses, emitted from an infected individual and existing on surfaces or in aerosols, can initiate infection in a person exposed to them. The other steps in this sequence are; i) confirming that live i.e. infectious virus is present and ii) confirming that sufficient live virus exists to initiate infection.

There are some limitations to this study. Firstly, there is an incomplete dataset for viral shedding as samples were not collected until 3 days post exposure in Donors and 2 days in Recipients. The primary outcome of the study was to confirm transmission; with this in mind we did not want to compromise transmission by performing sampling that may have interfered with the establishment of infection in subjects. Whilst there are no data to support such concerns, this was considered prudent. As a result it is likely that positive samples may have been missed. In addition, information about virus shedding patterns (particularly in Donors) is not available for the early stages of illness.

Secondly, the involvement in the quarantine phase of serologically immune subjects was unforeseen. All subjects were HAI negative at screening, for practical reasons this was performed up to 200 days in advance of the study. As a result subjects may have developed immunity between screening and quarantine (there was a range of between 86 and 143 days between these time points in the four immune subjects) so this period ought to be minimised in the future. In addition MN was not performed at screening.

Thirdly, we set an automatic start for the EEs, 44 hours after inoculation of Donors irrespective of symptom onset. Previous studies with the A/WI challenge virus showed maximal viral shedding on Day 2 and maximal

symptoms on Day 3 (personal communication: RVL). In the present study maximal symptoms also occurred on Day 3, but asymptomatic Donors needed to be randomly selected to take part in the EE on Day 2 because not enough were showing symptoms at this time. This led to Donors taking part who were subsequently found not to have been infected.

Finally, the fact that the study was conducted in early summer may not have provided optimal conditions for influenza challenge/transmission. It is interesting to note that high transmission rates of rhinovirus infection were seen in experiments conducted in the Antarctic (Holmes *et al*, 1976) and that responses to an influenza challenge differed between summer and winter; more symptoms and higher seroconversion rates were observed in winter (Shadrin *et al*, 1977). In the future, further consideration should be given to environmental conditions and the potential impact they have on infection and routes of transmission (Lowen *et al*, 2007; Shaman and Kohn 2009).

Whilst the proof of concept is achieved, we had stated in our objectives that a SAR of $\geq 25\%$ would be necessary to make subsequent studies using this model feasible and cost effective. There is potential to adapt the model to achieve a higher SAR in Recipients;

- Increase the number of Donor subjects either to ensure a minimum number with significant symptoms can be used in the EE or to increase the ratio of Donors:Recipients in an EE.
- Prolonging the EE would allow more time for transmissions to occur. Starting on Day 1 would allow an opportunity for pre-symptomatic Donor transmission to occur. Continuing through day 4 would allow Recipient exposure to Donors who develop symptoms later and those who have longer periods of viral shedding. Prolonging the exposure

time to Recipients has been observed to be important in rhinovirus challenge studies (Meschievitz *et al*, 1984). A disadvantage of prolonging the EE is that Recipient to Recipient transmissions could theoretically occur.

- Confirm infection and select Donors with the highest viral loads by using an influenza rapid test and/or PCR prior to the EE. High virus shedding in index cases has been cited as important for the infectiousness of rhinovirus (Fox *et al*, 1975; Meschievitz *et al*, 1984) and influenza (Ferguson *et al*, 2005; Cook *et al*, 2010), though it is interesting to note that symptoms alone in index cases have not been shown to be associated with transmission of influenza to household contacts (Viboud *et al*, 2004; Cauchemez *et al*, 2009a). Donors deemed to be the most infectious could be distributed across EE groups in an attempt to ensure that the infectiousness of Donors is evenly spread.
- More detailed immunological screening to assess susceptibility to infection (e.g. MN) and confirmation of susceptibility nearer to the start of the study.
- Performing the study in winter rather than summer time, or manipulate conditions such that temperature and humidity are favourable to virus survival and transmission.

Incorporating some or all of these modifications will have a number of implications for future studies. First, the generation of sufficiently high SARs in challenge models may require a departure from attempts to simulate 'normal' household conditions and interactions, for example using more Donors in the EE to overcome low SARs (which may be linked to the existence of minority populations of superspreaders). Whilst this might lessen the ability to translate findings to natural conditions, basic science

questions can still be addressed. Second, the use of more subjects will have logistical implications for staffing and space within a quarantine unit. Third, subject comfort must be considered during a 4 day EE, especially so if interventions are to be deployed to study routes of transmission. That having been said, it was evident that the subjects in the current study found that taking part in the EE was a highlight of their involvement. Finally, there will clearly be cost implications to use of more subjects, extra laboratory testing and manipulation of environmental conditions.

A major reservation about human challenge studies is whether experimental infection can be used as a surrogate for natural (wild type) influenza infection. Comparisons between the two must take account of the strains of viruses involved, levels of host immunity and the routes of infection initiation and transmission. We will now discuss some specific issues and consider what data is available or needed to address these reservations;

- Symptom severity with challenge studies involving nasal inoculation is generally perceived to be lower than with wild type infection, which in turn might affect the amount of viral shedding and reduce secondary attack rates. However, it is important to note that comparisons are often made with natural infections that present to medical services (Little *et al*, 1979); the clinical profiles of community cases (those who do not seek medical care) are probably more similar to those encountered during challenge studies (Carrat *et al*, 2002; Carrat *et al*, 2008; Lau *et al*, 2010). There is some evidence that inoculation by inhalation produces more severe symptoms than does intranasal inoculation (Henle *et al*, 1946) but this currently poses ethical constraints.

- Is the profile of viral shedding in challenge models comparable to wild type infection? Recent data that describes the time course and peak of viral shedding from community influenza cases (Lau *et al*, 2010) appear similar to those seen in experimental challenge studies (Carrat *et al*, 2008). More uncertainty concerns the generation of bioaerosol loads. It appears likely that experimental infections induced via intranasal inoculation might not cause as much lower respiratory tract disease. If this is reflected as reduced coughing and viral load in the LRT then the generation of infectious aerosols maybe less. This would have implications for the study of routes of transmission in challenge models.
- Questions can be raised about whether the relative contribution of droplet, aerosol and contact transmission might be different between experimental and wild type transmission and what impact environmental factors have in the different settings. There is no evidence for or against these issues at present although any potential differences could be overcome by carefully controlling the exposure conditions in experimental settings to mimic close household contact.
- Heterogeneity in influenza infectiousness likely exists and may be related to variation in symptoms and viral loads contained in respiratory sprays. However, we do not know what proportion of individuals exist as super-spreaders or how to identify them. Thus, the capacity to take account of such variability is limited, especially in studies that employ small numbers of subjects.
- The selection of subjects based on low levels of homotypic immunity (e.g. HAI titres) to the challenge virus helps to impart susceptibility to infection as occurs in natural infection. It is more difficult to assess heterotypic and T cell correlates of immunity (which may partly explain why not all inoculated subjects show clinical illness) but there is no

reason to suspect that a volunteer population would differ from the general healthy adult population in this respect. More detailed immunological screening for susceptibility would improve the efficiency of challenge studies but may have recruitment and cost implications.

If the utility of influenza challenge studies are to be maximised and the findings applicable to natural influenza infection there is not only a need to implement some modifications of study design but also to address the issues referred to above and a number of studies currently underway or planned will help to achieve this. A large prospective community study of influenza (Flu Watch) has taken place over the last 5 years and should provide detailed information on the symptoms of influenza experienced by community cases who do not usually seek medical attention (Hayward 2011). This data will allow a fairer comparison to be made with the symptoms experienced by experimentally infected subjects. Fluwatch is also collecting data on the immune correlates of protection against influenza infection. The composition of respiratory sprays (that include particles/aerosols) produced by subjects infected with influenza are being studied by investigators in the US (Lindsley *et al*, 2010b; Milton DK 2010) and this may provide data which can be used to address the issue on heterogeneity of infectiousness. Future challenge studies should attempt similar work with experimentally infected subjects.

5.6 Conclusion

Studying influenza transmission is difficult; seasonality, unpredictable attack rates, numbers of participants required, and confounding variables all present considerable obstacles to studying transmission of wild type infections. Experimental challenge infection can act as a proxy for natural infection and as such human influenza challenge studies could offer a promising approach to gain insights into both the mechanisms of influenza transmission and its prevention, so long as a reliable model of transmission can be developed. We demonstrate a successful 'proof of concept' for such an approach.

5.7 Acknowledgements

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Chapter 6:

Conclusions

6.1 Evidence Base

The literature review conducted as part of this thesis and conclusions drawn by international stakeholder organisations including WHO, CDC and IOM have identified a number of evidence gaps in our understanding of human influenza transmission. Broadly these gaps include the relative significance of the different routes of transmission, the efficacy of personal protective equipment to reduce transmission, the optimal deployment of non-pharmaceutical interventions such as school closures and appreciation of the factors that influence infectivity, including the molecular markers that promote transmission. In addition, they have highlighted the need for new and improved research methods.

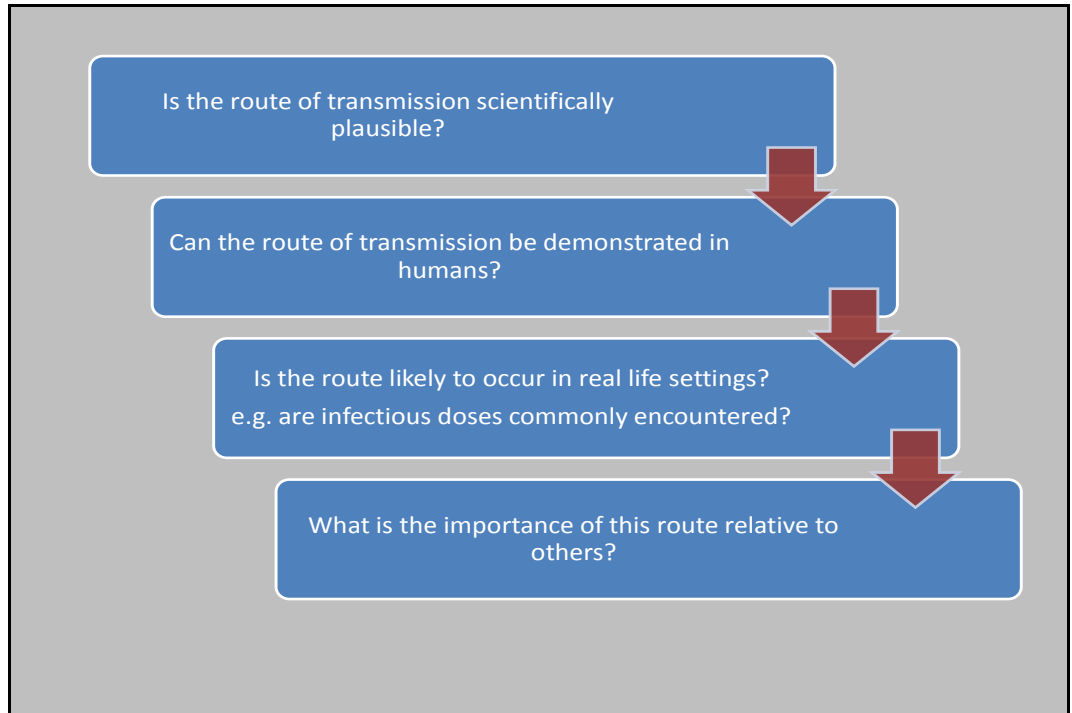
Understanding the routes of transmission and factors that influence them has been the focus of this thesis. This is an important area with major implications for infection control and public health policy. At present infection control guidance is based on relatively weak evidence and tends to be rather generic, adopting the “it can’t do any harm” principle rather than being based on knowledge that “it definitely matters” or “it definitely works”. Furthermore, considerable resources and finances are consumed during outbreaks of influenza as attempts are made to reduce spread and this is especially true of pandemics. Strengthening the evidence base to show which interventions work and when, is therefore vital to better protect public health and reduce unnecessary costs.

A series of postulates have been put forward to help establish whether proposed routes of microbial infection occur. These postulates are: 1) The infectious microorganism must be produced in the infected host at the proposed anatomic site of origin; 2) the organisms must be present in

secretions or tissue which are shed from the site of origin; 3) the microbe must be present and survive in or on the appropriate environmental substance or object; 4) the contaminated environmental substance or object must reach the proposed portal of entry; and 5) interruption of transmission by the proposed route must reduce the incidence of natural infection (Gwaltney and Hendley 1978). I have constructed a chain of evidence for influenza which follows the essence of these postulates. Research must progress down this chain to enable us to achieve a better understanding of influenza transmission (Figure 6.1).

Indirect evidence exists to suggest that all routes of transmission are plausible, but definitive answers to a number of key questions remain unanswered. For example; i) can a particular route be proven to occur? ii) is this route likely to occur in real life settings? and iii) what is the relative significance of this route compared to the others? Reasons for this include the fact that many studies have not sought to investigate routes of transmission as a primary objective and that fieldwork in natural settings, specifically assessing the dynamics and determinants of transmission amongst humans, has been limited. To move forward, a range of studies are needed to address and satisfy each level. Research involving humans is crucial as these are the only studies that can take the physical and social aspects of human behaviour and interactions into account. However, important contributions have been made and will continue to be made through laboratory, modelling and animal studies.

Figure 6.1: A chain of evidence to support the existence of routes of transmission.



This thesis contributes new data to the evidence base on routes of influenza transmission. Data on virus shedding and environmental contamination has been collected from infected patients in natural settings. It also proposes the human challenge model as a study design to investigate transmission; this follows the conduct of the first human to human influenza transmission study in modern times. In this concluding section, the findings and implications of my work will be considered in conjunction with the latest published material. Conclusions will then be drawn about our current understanding of transmission and the direction that future studies should take.

6.2 Shedding and environmental deposition of influenza virus

Findings in this thesis relating to nasal shedding and environmental deposition of influenza virus add to the debate about the relative significance of the routes of transmission and have relevance for both the timing and nature of infection control strategies.

6.2.1 Viral shedding

Viral shedding data provides estimates of the amount of virus present in the URT of an infected patient and can be monitored over time. This data can be used to assess infectious potential and in determining which routes of transmission may act. In general studies show that; i) viral shedding (detected by PCR) peaks early in the course of illness, typically over days 2-3 and lasts for approximately 6 days; ii) trends in viral shedding and symptoms over time are similar; iii) a proportion of individuals shed virus for prolonged periods. Factors associated with prolonged shedding include younger age (Cao *et al*, 2009; To *et al*, 2010), delayed antiviral use (Cao *et al*, 2009), major co-morbidity (Lee *et al*, 2009), hospitalisation (Leekha *et al*, 2007) and immunosuppression (Hayden 1997; Lee *et al*, 2009).

Despite these findings, it has not been shown that the duration of shedding equates to the infectious period. Two factors may help explain this. First, shedding when measured by PCR is unable to differentiate between viable and non-viable virus. Second, even when culture techniques detect viable virus, the ability of live virus to reach new hosts in an infectious dose cannot be guaranteed. Whilst there is some recent data to show that viral load (measured from the URT) is associated with infectiousness (Cook *et al*, 2009), it is possible that the different routes of transmission will depend on viral loads in both the URT and the LRT.

Future studies should seek to clarify the relationships between the duration and intensity of viral shedding with infectiousness. Index cases need to be identified and followed up on a daily basis with assessments of viral shedding and symptoms whilst secondary infections in their close contacts are captured to give a measure of their infectiousness. We should be looking for the social and biologic features that create a super-spreader. Such studies could be conducted in community or challenge study settings.

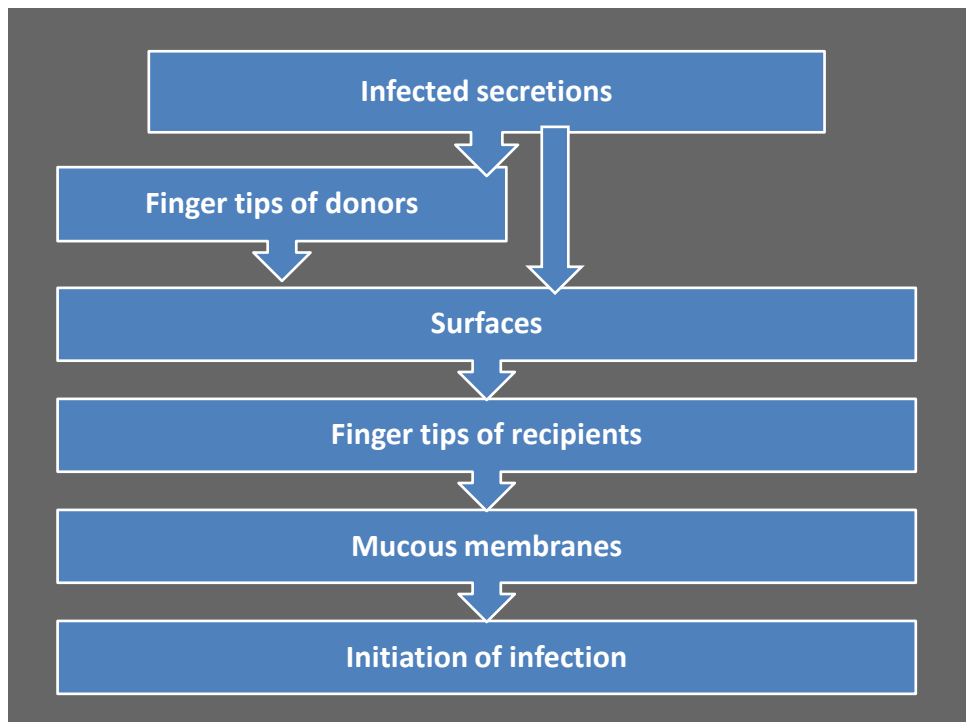
6.2.2 Fomite contamination

A role for fomites in the transmission of influenza appears widely accepted but limited data are available to directly support the possibility of contact transmission of influenza. Influenza can survive and remain viable on fomites long enough to permit transmission, however, the ability to survive does not necessarily equate to the ability to infect; sufficient virus must be transmitted to initiate infection. In support of the contact route of transmission are two studies that have shown significant effects of hand hygiene on the incidence of laboratory confirmed influenza and absenteeism due to ILI. In a study performed in Egypt by Talaat et al, an intensive hand hygiene programme was introduced to 30 schools over a 12 week period; 30 different schools acted as controls. In the control arm there were 0.5 episodes per 100 student weeks of absence due to an influenza-like illness (ILI), in the intervention arm the rate was 0.3. This gave a risk reduction of 40% ($p < 0.0001$). The incidence of laboratory confirmed influenza (both A and B) between the control and intervention group was also significantly reduced (Talaat *et al*, 2011). A study by Stebbins et al was conducted across ten primary schools in the US. Children in five intervention schools received instruction and hand sanitizers to promote hand and respiratory hygiene. Children who

developed an ILI over the course of the study (which lasted 6 months in total though testing was only performed over the latter 4 months) were tested for influenza. Although no significant effect of the intervention on the primary outcome (number of all confirmed influenza cases) was found, significantly fewer influenza A infections (incidence rate ratio 0.48; 95% CI: 0.26 - 0.87; $p < 0.02$) and school absence episodes (0.74; 95% CI: 0.56 - 0.97) were observed in the intervention schools. Interestingly influenza B infections were not reduced (Stebbins *et al*, 2011).

Despite the findings above, consideration of the transmission pathway for the indirect contact route does raise doubt about its plausibility. How likely is it that an infectious dose of virus can persist whilst passing along the transmission chain shown in Figure 6.2?

Figure 6.2: Chain of transmission for the indirect contact route.



A small number of studies have assessed and documented the presence of influenza virus on fomites around infected individuals. Work in this thesis found swab positive rates of 19% around experimentally infected subjects in a challenge study setting (Chapter 5) and 5.4% around naturally infected subjects (Chapter 4). Live virus was only recovered on two occasions. It is tempting to conclude that infectious virus does not contaminate the vast majority of surfaces but we must first be sure that our sampling and detection methods are fit for purpose; as discussed previously there is clearly room for improvement in this area (see Chapter 4 page 146). An interesting finding from Chapter 4 is that those who have high viral loads and the most prominent respiratory symptoms are the most likely to contaminate fomites. Such super-spreaders are the individuals most likely to initiate any successful chains of transmission.

New data on fingertip contamination comes from the second year findings of a study in Thailand. The fingertips of children with influenza were swabbed on Day 3 of their illness; over the course of 2 years 38 out of 191 (20%) children had positive swabs. The fingers of 9 out of 127 (7%) household contacts were also positive. Live virus could only be recovered from one swab (0.3%) (taken from an index case) (Suntarattiwong *et al*, 2011). The finding of virus on fingers of close contacts in this study does take us further down the chain but on only one occasion could live virus be detected and we do not know whether this represented an infectious dose.

I do not believe that the data currently available lend support to the idea that this chain of transmission plays a significant role in the spread of influenza. This mirrors conclusions drawn by investigators who have studied the indirect contact route of rhinovirus transmission. In a range of experiments they find that; i) transmission of infection via fomites under

'natural' conditions is difficult to achieve (Reed 1975; D'Alessio *et al*, 1984; Dick *et al*, 1987); ii) the transfer of virus from the start to the end of a transmission chain is negligible (Jennings *et al*, 1988); and iii) individual transfer steps were more efficient with inocula that were damp and contained a high viral titre (Reed 1975; Gwaltney and Hendley 1982; Jennings *et al*, 1988).

Further studies of fomite and fingertip contamination are needed to improve the evidence base but a necessary requirement is to improve the efficiency of sampling and laboratory techniques. To a greater or lesser extent, the transmission chain for the indirect contact route could be tested in a challenge model. An infectious dose of virus could be inoculated onto surfaces and the chain set in motion. Conversely, it should be possible to eliminate the contact route of transmission amongst individuals by preventing face touching. Rates of infection could then be compared between one group with face touching and another without; reduced rates in the no face touching group could imply that indirect contact transmission is operating in the other.

6.2.3 Air

Evidence backing up at least the potential for bioaerosol transmission of influenza is accumulating. Supporting evidence comes from the detection of influenza virus (by PCR) in the air of natural settings (Blachere *et al*, 2009; Lindsley *et al*, 2010a; Goyal *et al*, 2011; Yang *et al*, 2011), the demonstration of bioaerosol transmission in animal models (Mubareka *et al*, 2009; Munster *et al*, 2009) and mathematical modelling techniques which suggest a role for bioaerosol spread (Nicas and Sun 2006).

Detecting the presence of influenza in the air is the first step in a chain of evidence needed to confirm that influenza viruses, emitted from an

infected individual and existing as bioaerosols, can initiate infection in a person exposed to them. The other steps in this sequence are i) confirming that live virus is present and ii) confirming that inhaled live virus can initiate infection.

Findings in this thesis demonstrate for the first time that influenza can be detected in the air around identifiable influenza patients (as opposed to detection in buildings where multiple cases may contribute and to detection in exhaled breath captured directly into specialised devices). Recent work from other groups begins to take the evidence to the next level:

- Particle production from individuals with influenza has been assessed. Milton et al collected exhaled particles ($\geq 5\mu\text{m}$ and $0.05 - 4.9\mu\text{m}$) from 37 volunteers with seasonal influenza using a specially designed collection device. Sixteen out of 37 and 34 out of 37 subjects were positive for influenza in the larger and the smaller particles respectively. Virus numbers decreased rapidly between day 1 and day 2 of illness. Virus was detected by culture from two subjects (Milton et al, 2010).
- Lindsley et al collected cough particles from 47 volunteers with influenza; influenza was detected by PCR from 38 (81%) volunteers and 65% of the positive samples were from particles $\leq 4\mu\text{m}$ in diameter. Viable virus was isolated from 2 out of 21 samples tested. Significant heterogeneity between individuals in the amount of virus detected during coughing was observed. They also found that the amount of virus detected (by PCR) from nasopharyngeal swabs correlated well with the amount of virus found during coughing (Lindsley et al, 2010b). This is interesting because we assume that coughs generate a sample from the LRT whilst nasopharyngeal swabs

sample the URT. Furthermore, both this study and the one mentioned above found more virus in the smaller particles than the larger ones. This is counter intuitive as one might expect the larger particles to contain more virus as they make up the majority of the volume of a sample. A possible explanation is that the majority of virus shed into aerosols comes from the LRT, not the URT where larger particles originate.

- Aerosol inoculation of ferrets has been found to simulate natural infection more closely than intranasal inoculation and viable virus has been detected in exhaled aerosols (Gustin *et al*, 2011).
- Influenza has been detected in air samples obtained from around infected patients in a hospital setting. More virus was detected in the air from patients who had higher nasal viral loads. Furthermore 19% of patients (5 out of 26) were described as super-emitters of virus – a group who shed significantly more virus than the average (Bischoff, personal communication 2012).

As for fomite sampling, methodological limitations exist in detecting influenza in air samples. Collecting samples early in the course of illness, preserving virus viability (to allow culture) and the detection of low titres of virus present in air can be difficult. Sampling in well ventilated areas compounds this problem. These issues need to be considered in the design of future study protocols. Further study of the production of bioaerosols by infected individuals is needed, with identification of the traits associated with super-emitters. Work also needs to be done to confirm that individuals can transmit influenza via aerosols. Again, the challenge model could be used for such investigation; a) donors could be separated from recipients by a certain distance e.g. 2m so that the droplet and contact routes are eliminated; b) the aerosols from a room containing Donors

could be vented into a different room containing Recipients; c) an intervention could be deployed to only allow exposure to aerosols in one group whilst another group are exposed to all transmission routes – a face shield or a face mask that allow aerosols to pass could be used; or d) an intervention is deployed to reduce the amount of viable virus in aerosols, e.g. ultraviolet light. Again secondary attack rates could be compared between this intervention group and a control group. One must consider though whether the interventions used can interrupt SRAT.

6.2.4 Implications for routes of influenza transmission

I consider it likely that all routes of transmission have a role to play, but their relative significance will depend on a set of circumstances acting at a certain time. Dictating the process are factors related to the virus itself, the host and the environment. Transmission can likely occur through multiple routes in the same person; it is a dynamic and opportunistic process.

Data to back up the potential for aerosol transmission has been accumulating over recent years, but direct evidence is lacking. Factors that might promote transmission via aerosols include; the generation of bioaerosols by medical procedures, the existence of bioaerosol super-emitters, environmental conditions that favour the survival of virus in air and the presence of directional air flows and/or a lack of ventilation. High virus shedding probably increases the risk of transmission by all routes, but the potential for aerosol transmission may depend on it because the viral load per aerosol particle is small.

Direct evidence to support spread by droplets is also lacking. Assumptions that it plays a significant role have been based on epidemiologic

observations that transmission occurs at close range, but close range does not exclude a contribution from aerosols.

The data regarding contact transmission is difficult to interpret. Live virus is rarely found to contaminate hands or commonly touched fomites in natural settings but we must acknowledge that our ability to demonstrate this is constrained by methodological limitations. Intuitively, contact transmission will be more likely to occur when large volume, high titre inocula are deposited on fomites but the stochastic nature of the remainder of the transmission pathway makes infection difficult to envisage. In spite of this, indirect evidence in support of a role for contact transmission has recently emerged in studies that have used a hand hygiene intervention.

Circumstances are important in determining the route(s) of transmission that may occur. Consider some features that might be present in two common settings; hospitals and homes.

Hospitals:

- Patients requiring hospital treatment are likely to have severe symptoms and higher and/or prolonged virus shedding with an increased likelihood of transmission by all routes.
- Patients in hospital are likely to be relatively more confined than those in the community which will allow higher concentrations of virus to build up as aerosols and on surfaces. Confined settings are a feature of many outbreaks.
- A high number of susceptible individuals may come into contact with a hospitalised patient; e.g. HCWs, other patients, visitors, non-clinical hospital staff.
- Aerosol generating procedures are largely confined to hospitals.

- Engineering controls are more likely to be in place in hospitals; e.g. negative pressure ventilation rooms which will reduce aerosol loads. However, this is a limited resource and many patients are nursed outside of these areas. Natural ventilation, for example via windows, is often not possible in hospital rooms.

Homes:

- Peak viral shedding usually occurs early in the course of infection when individuals are more likely to be in their own homes, increasing the likelihood of transmission by all routes.
- Caregivers (family/friends) are often not vaccinated and do not usually have the use of personal protective equipment which could protect against transmission.
- Children in households are often identified as being transmitters.

6.2.5 Implications for infection control

An individual's infectious potential is related to the amount, dispersal pattern and duration of infectious virus release and their interaction with other susceptible individuals. The ability to identify those who are most infectious (super-spreaders) would be a major advance.

Influenza infection control isolation guidelines are largely based on data obtained during observations of households where most infection transmission appears to happen within 2 days of illness onset in an index case (Cowling *et al*, 2009b; France *et al*, 2010; Donnelly *et al*, 2011). Current guidelines from CDC recommend that "people with influenza-like illness remain at home until at least 24 hours after they are free of fever or signs of fever without the use of fever reducing medications". This guidance however, does not apply to health care settings where "the isolation period should be continued for 7 days from symptom onset or

until the resolution of symptoms, whichever is longer" (CDC 2009a). This reflects data on shedding from hospital cases and the fact that the hospital houses a vulnerable (to the complications of influenza) population. It is important that guidelines are practical and adaptable. At the beginning of the 2009 pandemic when data on the dynamics of transmission and severity of disease were sparse, CDC guidelines recommended that an isolation period of 7 days be observed in all cases. However, as data became available the recommendations were altered. The impact that recommendations have on the community at large must be considered, especially during a pandemic. Days off work, particularly for those involved in the delivery of essential services (e.g. emergency services, energy supplies) because of enforced isolation, including the need to take time off to care for others, need to be safely minimised.

Viral shedding data provides a less useful approximation of infectiousness, especially if based on PCR, primarily because our knowledge of what constitutes an infectious dose is lacking. Knowing that live virus can be shed from cases for 5 days and that virus may persist in the environment for up to 2 days does not necessarily mean that infection can be transmitted for up to 7 days; only the presence of an infectious dose would validate this statement. Viral shedding data might have more use in determining risk of transmission if one could show that a relationship between the amount of virus shed and the risk of infection existed. Measures other than conventional nasal viral shedding need to be further explored, e.g. 'shedding' of virus in association with a cough or sneeze and the physical properties of particles released i.e. those which settle quickly and those which remain suspended in air as bioaerosols. This might allow for the identification of individuals who release large amounts of virus into the environment, so called super-emitters. A study of rhinovirus infected

patients found that just under a quarter of individuals were responsible for over 80% of the exhaled particle load (Fabian *et al*, 2011). Super-emitters may in turn (if they are shown to be more infectious than others) become super-spreaders. The existence of super-spreaders would have profound implications for our understanding of influenza transmission and for control strategies. High heterogeneity in infectiousness implies that relatively few individuals generate most of the transmission — or conversely, that many individuals hardly transmit at all. The practical implications of this would be that control efforts should aim to identify highly infectious super-spreaders (and circumstances that favour their creation) and target vaccination or other interventions at them.

There are several other issues related to infection control practice where the route that transmission takes might impact upon guidance:

- What is the safe distance from an infectious case?
- What interventions should be used to reduce transmission?
- Which medical procedures generate significant bioaerosols?
- Can influenza transmit through the conjunctiva?
- What advice about interventions should be given to the general public?

Some evidence is emerging to help refine infection control practice, for example the delineation of medical procedures that are aerosol generating, but at the same time the emergence of other evidence, e.g. the implication of the aerosol and/or conjunctival routes of transmission, might dictate significant changes. At present, with limited evidence on routes of transmission, we still rely on historical perspectives and common sense for many recommendations.

6.3 Human challenge studies

Human challenge studies offer the potential to investigate various aspects of transmission. As well as assessing the effectiveness of interventions to reduce transmission (which may be used to make inferences about routes of transmission), they also provide the opportunity to address questions about virus survival in the environment, the effects of temperature and humidity on survival/transmission and the effectiveness of engineering controls e.g. ventilation and UV light to reduce transmission. In addition, it could also be possible to study both pre-symptomatic and asymptomatic influenza infection and how transmission is affected by the presence and degree of specific symptoms. The generation of such information is fundamental to better understanding routes of transmission and their relative significance.

Whilst there are clear advantages to using an experimental human challenge model, there are also a number of hurdles to overcome. The advantages of the model include; i) the provision of symptomatic subjects at a specific time; ii) the ability to control subject's behaviour and environment; iii) viral shedding and symptoms can be recorded prospectively; iv) the numbers of subjects needed for adequate powering of studies is greatly reduced; v) the studies are repeatable and can be conducted at convenient times; and vi) pre-screening is possible to assess susceptibility to infection based on serologic testing, this increases study efficiency and in immunological terms is the closest one can get to a pandemic situation.

The drawbacks of the challenge model include the facts that; i) the development of a satisfactory challenge virus, manufactured in accordance with GMP is not straightforward; ii) although the viruses used are closely

derived from wild-type, they tend to be less virulent than most naturally occurring influenza infections that present to medical services because the nasal route of administration affects illness severity. This in turn may have an effect on infection transmission between humans; iii) creation of symptomatic illness in humans using a GMP challenge virus is well proven but a successful and robust model of person-to-person transmission with a GMP challenge virus has yet to be established; iv) transmission would need to occur above a given rate to enable the practical design of follow on studies, for example a low secondary attack rate would mean that larger numbers of volunteers are needed to adequately power the studies. Although more efficient in numbers than a community study, the numbers required for a randomised intervention study are still relatively large; v) given the quarantine and safety features required for these experiments the costs are considerable; vi) the recreation of natural, e.g. household conditions, in a quarantine facility is challenging.

The proof of concept study (Chapter 5) demonstrates for the first time that transmission can be achieved following infection initiation with a challenge virus in a quarantine setting. Modifications to the study design may allow the development of a reliable model where secondary attack rates are sufficient to allow logistically feasible and cost effective studies to take place.

Groups in the US ceased to use the influenza human challenge model approximately 10 years ago; reasons included the lack of availability of new challenge viruses and the occurrence of a serious adverse event during a trial. This concerned a subject who developed myocarditis following experimental challenge with influenza, but who recovered fully. A subsequent study to investigate cardiac findings during natural influenza

infection revealed that although ECG abnormalities were common, they were also clinically insignificant and resolved promptly without intervention (Ison *et al*, 2005). Safety remains of paramount concern, and an extensive review of the literature (covering a total of 56 different studies with 1,280 healthy participants) which concludes that experimentally induced infection is a mild disease is therefore reassuring (Carrat *et al*, 2008).

A balance must be struck when performing influenza challenge studies between producing infection of realistic severity compared with wild type infection, whilst on the other hand demonstrating an acceptable safety profile that permits ethical approval. Some compromise of the former is inevitable and occurs through the use of a well characterised challenge virus and the intranasal route of inoculation as opposed to the aerosol route which has been associated with more severe disease. Guidance on the conduct of microbial challenge studies in humans in the UK was issued in 2005 (The Academy of Medical Sciences 2005) and a review of the ethics of infection inducing challenge experiments suggests that the following questions be asked when evaluating studies (Miller and Grady 2001);

- Is the scientific rationale for using a challenge model acceptable?
- Are the risks of challenge studies acceptable?
- Are the discomforts of challenge experiments acceptable?
- Does the study enrol subjects from vulnerable populations?
- Does the informed consent process adequately inform potential subjects about the risks and discomforts?
- Does the amount of financial compensation offered to volunteers constitute undue inducement?

Given the positive outcome of the proof of concept study, as long as ethical and safety concerns continue to be addressed and satisfied, the challenge model could have an important role to play in the study of human influenza transmission.

6.4 Looking ahead

Based upon the all the work considered and presented in this thesis, I judge the following to represent important gaps in our knowledge with regard to influenza transmission and where future research efforts should be directed;

- Heterogeneity of infectiousness; there is a need to confirm the existence of super-spreaders of influenza infection, understand the factors involved and learn to identify such individuals. An understanding of the contribution made by an individual's physiology, behaviour, symptoms and viral loads in the upper and lower respiratory tracts in relation to the amount of virus emitted in respiratory sprays is needed.
- Infectious dose(s) needed to initiate infection via the different routes. Whilst some data from experimental challenge models is available, the methods and routes of inoculation differ from natural infection. Sound estimates of the infectious doses for each route would substantially improve the outputs of bio-mathematical transmission modelling.
- The effect of variations in temperature and humidity.
- Viral determinants of transmission; the ability to predict the transmissibility of a virus, e.g. a future pandemic virus, would be invaluable.

- Virus emission and deposition data from infected patients and their near environments.
- Methods and technologies to reliably sample, detect and quantify influenza virus.
- The risk of transmission from asymptomatic and pre-symptomatic individuals.
- Transmission distances; both close and prolonged contact with infectious cases are often identified as risk factors for transmission. Aerobiology suggests that droplet transmission is unlikely to occur beyond 2m because droplets fall out of the air before reaching this distance. There is little evidence for long range transmission by aerosols and although safe distances are not known the risk is expected to fall as distance from a source increases.
- Efficacy and effectiveness of interventions e.g. face masks, UV light; in healthcare settings the main debate concerns the relative importance of aerosol transmission and whether respirators should be used instead of SFMs. Two randomised controlled trials have failed to show a benefit of respirators over SFMs. If it were possible to identify the circumstances in which aerosol transmission becomes likely then a range of mitigation strategies could be employed. These might include the use of a respirator, administrative (isolation rooms, movement restriction) and engineering controls (UV light, negative pressure rooms, manipulation of humidity) and prescription of saline nebulisers to index cases to decrease aerosol production (Edwards *et al*, 2004). Currently the only circumstance where respirators (as opposed to SFMs) are recommended for protection against influenza in the UK is during the conduct of aerosol generating procedures. Recommendations may change however, if the risk vs. cost vs. benefit assessment alters such as may be the case with a virulent virus.

- Aerosol generating procedures; better characterisation of AGPs will improve risk management. For example a study has recently shown that chest physiotherapy and non-invasive ventilation are droplet (not aerosol) generating procedures (Simonds *et al*, 2010).
- Transmission via the conjunctiva; the recent finding that the eyes permit the passage of bioaerosols to the nasopharynx raises the question of whether eye protection is a necessary measure (Bischoff *et al*, 2011).

In order to address these questions a variety of studies with different designs are required, no one study will be able to provide all the answers. Basic science laboratory based research is necessary to underpin and direct research in the field; outbreak investigations may be able to focus more on whether particular circumstances favoured specific routes of transmission and could look for evidence of super-spreading events; animal models are providing insights into the viral determinants of transmission and the effects of environmental conditions; and it is hoped that the generation of new and reliable data can be fed into increasingly sophisticated transmission modelling approaches. An exciting prospect is that of experimental challenge studies which may be able to overcome many of the problems faced by other studies (Killingley *et al*, 2011a). They could be used to demonstrate that specific routes of transmission occur by selectively blocking others, measure the effectiveness of intervention strategies, assess the effects of environmental conditions and be used as a rich source of data for measurements of viral shedding and deposition. However, studies of patients with natural infection and in natural settings will remain the gold standard in many respects and provide reference points for data obtained during experimental challenge studies. Intervention studies in natural settings will continue to have an

important role and are likely to be more successful in the future following reflections on those conducted to date (Aiello *et al*, 2010a; Klick *et al*, 2011).

The main challenge when it comes to conducting research in natural settings is to be able to gather data in a timely manner. As peak viral shedding and infectiousness usually occurs within 2 days of illness, being able to recruit sufficient numbers of subjects and collect data or execute interventions within this time frame is critical. Unfortunately the majority of studies done to date [including the study presented in this thesis (Chapter 4)] have not always been able to meet this challenge. The usual method of recruitment has been to enrol cases that present to medical services, but presentation is often delayed, and even if recruitment occurs within 48 hours the time to intervention or data collection is longer. An alternative approach which can overcome this problem is to follow up a cohort of individuals recruited before illness begins, but this is far more resource intensive. Innovative recruitment approaches to potential patients (that are approved by ethics committees) are needed. Use of social media, advertising and publicity before and during an influenza season should be explored.

To move forward and meet the challenges addressed above, those involved in influenza transmission research and those concerned with public health and infection control policy must work together to develop a research agenda that will lead to a strengthening of the evidence base. We should then be better able to mitigate the impact of influenza in the future.

Closing Remarks

In the early years of my specialist registrar training programme, performing research was often talked about and aspired to, and something which many of my more senior colleagues were doing. I must admit though that it didn't seem particularly relevant or attractive to me at that time; I was too busy and caught up in my clinical training and I derived all my satisfaction and drive from looking after patients. As my training progressed however, the attraction of academia grew. Taking on a new challenge was part of it, but there was also a growing realisation that there was more to being a good doctor than just treating the patient in front of you. Being able to influence and improve the quality of care provided to a population of people seemed to be the next step up. It is not just through research endeavour that such improvements are made. Asking questions and pushing boundaries are important, and as these became more tangible to me I wanted to get involved. It also became clear that the skills one develops and acquires through conducting research would help make me a better doctor, more able to positively engage with the healthcare system at large.

It was during my third year that I began to consider what research options might be available. A six month secondment at the Department of Health, working within the infection control team, raised my awareness of hospital associated infections and reflecting on my own clinical experiences I became interested in the use of face masks to prevent the spread of infection. At the time (2006) influenza pandemic preparedness planning was well underway, but the role that face masks could play in this was uncertain. At this point I knew that I wanted to undertake clinical as opposed to laboratory based research as I didn't want to lose contact with

patients. I had some initial discussions with prospective supervisors and it was Professor Jonathan Van-Tam who showed both enthusiasm and commitment towards me and who was poised to start up a research group in Nottingham with interests aligned to mine. It took some time to develop and build a line of enquiry that would satisfy a PhD but this we did and the funding from the MRC that followed was the green light to make things happen.

I have thoroughly enjoyed the journey that completing this PhD has taken me on. As I reflect on this, two things stand out as having contributed to my personal development. The first is the people I have encountered along the way; national and international influenza experts; staff at agencies such as the Department of Health, WHO, CDC; colleagues at the University of Nottingham, volunteers involved in my studies, collaborators from other academic institutions and all those involved in the research process from ethics committees to research nurses. Learning to interact and to work with such people has been an important experience and the contacts made and friends forged will remain invaluable throughout my career.

The second has been my exposure to the research process. Research plays a key role in continually improving healthcare. Having an appreciation of what's involved, e.g. applying for funding, setting up a project, working with ethics committees, adhering to the principles of GCP and research governance will again be invaluable as my career progresses and will allow me to interact positively and usefully with colleagues from all disciplines.

The topic of influenza transmission although close to my heart is perhaps of secondary importance; it's the personal development journey that has

been key. Meeting new people, learning new skills and gaining an experience of academic work will serve me well in my future career, whatever path that takes.

Glossary

A(H1N1)pdm09 - The pandemic A(H1N1)2009 virus has become a seasonal influenza virus, continuing to circulate with other seasonal viruses since August 2010 when the World Health Organization (WHO) declared the end of the influenza A(H1N1) 2009 pandemic. However, the nomenclature of this virus has never been standardized resulting in the use of diverse names for the same virus. In order to minimize confusion and to differentiate the virus from the former seasonal A(H1N1) viruses circulating in humans before the influenza A(H1N1) 2009 pandemic, the advisers to the WHO technical consultation on the composition of influenza vaccines for the southern hemisphere 2012 season advised WHO to use the nomenclature above (WHO 2011b).

Aerosol - A gaseous suspension of fine solid or liquid particles. An aerosol can consist of a range of particle sizes; small particles will remain suspended in the air for prolonged periods of time (droplet nuclei) whilst larger particles (droplets) will quickly settle to the ground. In this review the term aerosol transmission will refer to the transmission of infection by droplet nuclei only.

Aerosol transmission - Transmission of influenza through the air by droplet nuclei (particles <10µm). Particles are respired and penetrate proximal airways to reach the lung where they can initiate infection.

Airborne - Carried by or through the air.

Bioaerosol - A gaseous suspension of fine solid or liquid particles that are living, contain living organisms or were released from living organisms.

Contact transmission – the transfer of an infectious agent from one being to another by touch;

- Direct Contact – transmission via direct physical contact; for example a kiss.
- Indirect Contact – transmission via intermediate objects (fomites) e.g. door handle or hand.

Droplet – A particle $>20\mu\text{m}$ and $<500\mu\text{m}$.

Droplet nuclei – A particle $<10\mu\text{m}$.

Droplet transmission - Transmission of influenza through the air by droplet particles ($>20\mu\text{m}$) emitted by an infected host (e.g. by coughing) which deposit on mucous membranes either directly or by inhalation.

Face mask (medical or surgical) – A protective covering for the mouth and nose. Whilst it provides a physical barrier to large projected droplets, it does not provide full respiratory protection against droplet nuclei.

Fomite - An inanimate object or substance capable of carrying infectious organisms.

Inhalable - Particles that enter the body through the nose and/or mouth during breathing. They do not travel further than the tracheobronchial tree.

Respirable - Inhaled particles that penetrate to the alveolar region of the lung.

Respirator - A protective covering for the mouth and nose. It provides a high level of filtering capability and face fit

- FFP2/N95 – respirators that are able to filter out particles of $>0.3\mu\text{m}$ with an efficiency of 95%
- FFP3/N99 – respirators that are able to filter out particles of $>0.3\mu\text{m}$ with an efficiency of 99%

FFP 2/3 (Filtering Face Piece) is a European classification system whereas as N95/99 is the US equivalent (although testing protocols are not identical and the ratings are not directly interchangeable).

Appendices

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Chapter 4

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Chapter 5

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- Appendix 5.8 Serology results by laboratory

Appendix 2.1: Summary of studies that inform the biologic plausibility of the proposed routes of transmission

Author (Year)	Study / Investigation	Main Findings
Bean (1982)	Virus survival on surfaces and hands	Virus survives for 24-48 hours on hard surfaces, 8-12 hours on soft surfaces and for up to 5 minutes after virus transfer to hands. The authors conclude that a person shedding large quantities of virus ($>10^{5.0}$ TCID ₅₀ /mL) could transmit infection via stainless steel for 2 hours and via tissues for a few minutes.
Thomas (2008)	Virus survival on banknotes	Survival time of viruses on banknotes was directly related to inoculum size. The addition of respiratory mucus to inoculums increased the duration of infectiousness, e.g. 8 days vs. 2 hours for an H3N2 virus. The authors then went on to show that virus contained in nasopharyngeal secretions obtained from ill children survived on banknotes for at least 24 hours in 50% and at least 48 hours in 36%.
McDevitt (2010)	Virus survival on steel and the effect of temperature and humidity	Influenza virus survival is affected by temperature, relative humidity (RH) and exposure time after being deposited on a stainless steel surface. Drying in ambient conditions (temperature 24°C, relative humidity 35%) for an hour resulted in a reduction of 63%. It was shown that viral inactivation increased with rising temperature (55 → 60 → 65°C) and RH (25 → 50 → 75%).
Greatorex (2011)	Virus survival on household surfaces	Virus survival on a range of representative household surfaces was studied. Viable virus could be recovered from most surfaces 4 hours after inoculation although differences between porous (less survival) and non porous surfaces were evident. However, viable virus could not be detected on any surface other than the plastic (Petri dish) control 9 hours after inoculation. Similar results were found when a 2009 pandemic H1N1 strain was tested.
Thomas (2010)	Virus survival on hands	Viable virus could be detected on fingertips for up to 30 minutes. Bigger volumes of inoculum led to more virus being detected and it was shown that if the viral inoculum was spread on the fingertip (rather than being left as a drop) survival time was less.
Grayson (2009)	Virus survival on hands	A relatively high dose of an H1N1 virus (10^7 TCID ₅₀ /0.1mL) was used to contaminate the hands of 20 volunteers. After 2 minutes, a reduction in virus as measured by PCR was seen and virus was cultured from the fingertips of 14 volunteers (a 3-4 log reduction in virus TCID ₅₀ was seen). Eight volunteers were assessed after 60 minutes; little further reduction in virus levels (assessed by both culture and PCR) was seen.

Simmerman (2010)	Virus detection on hands during natural infection	The hands of infected children and secondary cases within households were swabbed. The hands of 15/90 (16.6%) index cases were positive by PCR, one (1.1%) was culture positive, whilst 1/59 (1.6%) secondary cases were PCR positive and none were culture positive.
Simmerman (2010)	Virus detection on fomites during natural infection	540 swabs were collected from fomites in households with an index case. 3% were positive by PCR and 17.8% households had at least one positive swab. No swabs were culture positive. Households in which the index case was <8 years old had a significantly higher prevalence of contamination.
Killingley (2010)	Virus detection on fomites during experimental infection	Fomites were swabbed during a study that involved subjects who were experimentally infected with an H3N2 influenza virus. 9/48 swabs (19%) taken from a subjects' rooms revealed influenza (by PCR); no live virus was found.
Killingley (2010)	Virus detection on fomites during natural infection	704 swabs were collected from fomites in the homes and hospital rooms of confirmed influenza patients as part of a study during the 09/10 and 10/11 seasons. Virus was detected by PCR on 25 occasions (3.5%). Live virus was recovered from two surfaces.
Weber (2008)	Review: Influenza survival in aerosols	A number of authors have attempted to measure the survival of influenza virus in air and most find that survival is prolonged (up to 24 hours) at low RH.
Fabian (2008)	Detection of influenza in aerosols from patients	Patients with influenza were asked to directly breathe into a device that collected filtered samples and employed optical particle counting and airflow data. Influenza was detected by PCR in 4/13 samples collected.
Lindsley (2009 and 2010)	Detection of influenza in aerosols in medical care facilities	Air samples were collected from urgent care medical facilities during 2 influenza seasons. Both stationary and personal samplers collected air particles containing influenza A virus.
Milton (2010)	Detection of influenza in aerosols from patients	Exhaled breath particles were collected from 37 volunteers with seasonal influenza using a specially designed collection device. PCR and culture were used to detect virus. Virus numbers decreased rapidly between day 1 and day 2 of illness. Virus was detected by culture from two subjects.
Tseng (2010)	Detection of influenza in aerosols	Air samples were collected from a paediatric department and aerosol fractions were found to contain influenza (by PCR).
Lindsley (2010)	Detection of influenza in aerosols from patients	Cough particles from 47 volunteers with influenza were collected; influenza was detected by PCR from 38 (81%) volunteers, 65% of the particles were of a respirable size and viable virus was isolated from 2/21 samples tested. Significant heterogeneity between individuals in the amount of virus detected during coughing was revealed.

Yang (2011)	Detection of influenza in aerosols	Air samples were collected from a health care facility, a day care centre and aeroplanes. Based on the concentrations of virus found a model is created which finds that over an hour enough virus can be inhaled to induce infection. NB the amount of virus was quantified by PCR which may not reflect the amount of infectious virus present.
Bischoff (2011)	Demonstration of the ocular transmission route	Volunteers were exposed to an aerosolised live attenuated vaccine influenza virus. When only the eyes were exposed, virus could be detected 30 minutes later from the nasopharynx. Infection itself was not demonstrated (this was not the aim) but it does appear that nasolacrimal ducts can transport virus to its target cells.

Appendix 2.2: Summary of studies examining the epidemiology of disease in closed or semi-closed settings

Author	Setting	Virus (year)	Special features / identified risks	Likely dominant route(s) of transmission
Blumenfeld	Hospital ward	H2N2 (1957)	Pandemic virus	All routes possible
McLean	Hospital Ward	H2N2 (1957)	UV light, pandemic virus	Aerosol
Moser	Aircraft	H3N2 (1977)	Point source, no ventilation	Aerosol
Klontz	Barracks and aircraft	H1N1 (1986)	Outbreak amongst a squadron	All routes possible
Morens	Nursing Home	H3N2 (1989)	High level care patients	Contact
Cunney	Neonatal Unit	H3N2 (1998)	Twins, mechanical ventilation	Contact / Droplet
Awofeso	Prison	H3N2 (2000)	Infection introduced into a closed community	All routes possible
Han	Tour group + aircraft	H1N1 (2009)	Talking with index case, pandemic virus	All routes possible
Baker	Aircraft	H1N1 (2009)	Pandemic virus	All routes possible
Apisarnthanarak	Hospital ward	H1N1 (2009)	HCW providing direct care, pandemic virus	Contact / Droplet
Wong	Hospital ward	H3N2 (2008)	Aerosol generating procedure and airflows	Aerosol
Cui	Train	H1N1 (2009)	Close contact and prolonged exposure associated with transmission	All routes possible
Piso	Bus	H1N1 (2009)	Very low secondary attack rate	All routes possible
Magill	Hospital	H1N1 (2009)	Transmission amongst HCWs significant	All routes possible

Appendix 2.3: Summary of prospective non-pharmaceutical intervention (NPI) studies

Study (Year)	Study design	Study aim (n=subjects analysed)	Study setting / randomisation unit	Study arms	Main findings
Talaat (2010)	Cluster RCT	Primary prevention (n=44451)	Schools	Hand hygiene / control	Significant reductions in ILI absenteeism and lab confirmed influenza (A+B)
Stebbins (2011)	Cluster RCT	Primary prevention (n=3360)	Schools	Hand + Respiratory Hygiene / Control	Significant reductions in absenteeism and lab confirmed influenza A
Aiello (2008)	Cluster RCT	Primary prevention (n=1297)	University residences	SFM / hand hygiene + SFM / control	No difference in cumulative incidence of ILI. During study weeks 4-6, weekly ILI incidence reduced in SFM + hand hygiene vs. control
Cowling (2009)	Cluster RCT	Secondary prevention (n=794)	Households (including index cases)	Hand hygiene / SFM + hand hygiene / control	No difference in lab confirmed SAR between study arms. Some effect seen if interventions (hand hygiene + SFM) implemented within 36 hrs
MacIntyre (2009)	Cluster RCT	Secondary prevention (n=286)	Households	SFM / respirator / control	No difference in rate of ILI between arms. If compliant with mask use reductions in ILI seen with both masks
Larso (2010)	Block RCT	Primary and secondary prevention (n= 2788)	Households	hand hygiene / hand hygiene + SFM / control	No difference in rates of clinical infection (upper respiratory infections and influenza). SFM use associated with reduced SARs
Simmerman (2011)	RCT	Secondary prevention (n=887)	Households	Hand hygiene / SFM + hand hygiene / control	No difference in lab confirmed SAR between study arms
Loeb (2009)	RCT	Comparative non-inferiority (n=446)	HCWs (in hospitals)	SFM / Respirator	SFMs were non-inferior to respirators in relation to rates of lab confirmed influenza
MacIntyre (2011)	Cluster RCT	Comparative efficacy (n= 1441)	Hospitals (HCWs)	SFM / Fit tested Respirator / Non fit tested respirator	Respirators were associated with an approximate halving of risk for all infection outcomes compared to SFMs, but after adjustment for clustering, the only significant finding was that non-fit-tested respirators were more protective against clinical respiratory infection.

Appendix 2.4: Summary of human influenza challenge

Author (year)	Study / Investigation	Main Findings
Henle (1946)	Experimental infection	Findings from over 200 volunteer exposures identified the route of inoculation as important; infection by inhalation led to fever much more frequently than did nasal instillation (89% vs. 13%).
Alford (1966)	Experimental infection initiated via aerosols	23 volunteers inhaled 10 litres of an H2N2 aerosol delivered via a facemask. The dose of virus delivered ranged between 1-126 TCID ₅₀ . Four volunteers developed clinical illness; virus was isolated from these and one other volunteer, whilst seroconversion was seen in seven including all those who exhibited illness. Noting limitations of the study design and making an assumption that only 60% of the aerosol load inhaled will reach the lower respiratory tract the study reports that half of the volunteers with very low pre-existing antibody titres were infected with 0.3-6 TCID ₅₀ .
Little (1979)	Comparison of natural and experimental influenza	Natural infections produced more fever, more cough and had a more marked effect on pulmonary function tests.

Appendix 2.5: Summary of animal transmission studies

Author (date)	Study / Investigation	Main Findings
Andrewes (1941)	Experimental infection in ferrets	Transmission occurred between ferrets housed in different cages and separated by distances that would arguably only permit aerosol spread.
Schulman (1968)	Experimental infection in mice	Transmission was demonstrated between mice housed in the same and separate cages and the frequencies of transmitted infection were similar. One experiment allowed the ventilation in a cage housing Donors and Recipients to be altered; when ventilation was increased, infection rates decreased. These findings were interpreted as signifying that aerosol transmission was active.
Lowen (2006)	Transmission in guinea pigs	A human H3N2 virus was shown to replicate well in guinea pigs after intranasal inoculation and transmission from infected to recipient animals occurred when animals were housed together or in separate cages (side by side and separated by 91cm).
Lowen (2007)	Investigation of the effect of humidity and temperature on transmission in ferrets	Experiments on guinea pigs housed in an environmental chamber were conducted that only allowed for droplet or aerosol transmission. Low RH (20-30%) seemed to favour transmission while higher RH (80%) inhibited it. In another set of experiments transmission occurred at low temperature (5°C) more frequently than higher temperatures (20 and 30°C).
Lowen (2008)	Investigation of the effect of temperature on contact transmission in ferrets	Recipient guinea pigs were placed in cages that had housed infected ones with ambient temperatures of 20 and 30°C. Transmission was seen to occur equally at both temperatures; the authors suggest that whilst droplet and aerosol transmission is reduced by high temperatures, contact transmission is not (as virus can be passed directly between animals it is not exposed to the outside environment).
Mubareka (2009)	Routes of transmission in guinea pigs	The relative contributions of droplet/aerosol and fomite (contact) transmission were studied. Infected and recipient animals were placed in separate cages >80cm above each other and transmissions occurred. However, when recipient animals were placed in the cages of infected animals (infected animals were removed but fomites were not) less infection transmission was seen.
Belser (2010)	Review of molecular determinants of transmission	The importance of the haemagglutinin and polymerase proteins in influencing transmission are highlighted
Gustin (2011)	Comparison of aerosol and intranasal inoculation of ferrets	An aerosol inoculation system was devised. Aerosol inoculation caused a more natural influenza infection. Onward transmission was dependent on the level and duration of virus shedding. Viable virus was detected from infected ferrets in exhaled aerosols.

Appendix 2.6: Summary of modelling investigations

Author (date)	Study / Investigation	Main Findings
Atkinson (2008)	Model to quantify role of aerosol, contact and droplet routes based on a household scenario	They found that; i) most transmissions occur early in an infected person's illness, in fact over half occur in the pre-symptomatic period; ii) a caregiver is twice as likely to be infected than a non-caregiver; iii) a very small proportion of virus exits on small aerosol particles; iv) virus survives in air longer than it does on hands and v) the infectious dose for virus in aerosols is much smaller than that in either droplets or settled particles. The model leads them to conclude that aerosol transmission is far more dominant than contact transmission. With regard to droplet transmission, they find that a well directed cough or sneeze carries an infection probability of 0.011 and 0.981 respectively, though it must be stated that a well directed cough or sneeze may be a rare event.
Nicas (2008)	Investigation of the hand to face contact route of transmission	The scenario was a caregiver attending a sick family member in a bedroom for 30 minutes. An infection risk due to hand contact of 0.011% was generated.
Nicas (2009)	Model to quantify routes of transmission based on a scenario of visiting a patient's room	Important variables were considered to be i) infectious doses and ii) viral titres. When the URT:LRT infectious dose ratio is 3200:1 contact, aerosol and droplet routes all contribute substantially to infection risk. With rising viral titres contact and aerosol become more significant. When the ability of virus to reach target cells is taken into account aerosol transmission assumes dominance.
Wagner (2009)	Risk assessment of aerosol transmission aboard an airplane	The authors find that proximity and duration of exposure to the source and passenger density are important factors. Up to 17 infections could be caused during a 17 hour flight.
Spicknall (2010)	Model to quantify routes of transmission	The indirect contact mode of transmission appeared dominant. However, the authors explain that this is not necessarily the case in all settings. Out of 10,000 model runs indirect contact was dominant in 3079, respiratory in 121 and droplet in 66. Furthermore, considerable overlap is also seen where modes appear co-dominant, this occurred in 1969 sets. Further analyses were then performed to identify specific parameters that determine transmission intensity; these included infectious doses, shedding magnitude (amount of virus present in the environment) and host density.
Myatt (2010)	Model to assess the effect of humidity on virus survival	Based on 2 storey house, portable humidifiers could potentially increase absolute and relative humidities to achieve reductions in airborne influenza virus survival of up to 31% in a single room and 14% in the entire house.
Teunis (2011)	Model to quantify routes of transmission	Infectious dose data from influenza challenge studies are used in the construct of a model that simulates infection from a patient in a poorly ventilated room. Infection droplets and aerosols are approximately equal.

Appendix 2.7: Summary of evidence for routes of influenza transmission

Evidence	Indirect Contact	Droplet		Aerosol
		Direct Contact	Inhalation	
Plausibility	Virus can survive on fomites and hands but transmission must overcome several hurdles to occur and because of this the process appears tenuous. Few data are available to show viable virus exists on hands or fomites in natural conditions	Virus is present and can survive on droplets. The bigger the droplet the higher the titre of virus that can be present. This is important as the URT requires a high infectious dose. However, the chance of a droplet reaching its target cell is low	Virus is present and can survive on droplets. The bigger the droplet the higher the titre of virus that can be present. This is important as the URT requires a high infectious dose. However, it would require a perfectly directed cough or sneeze to enable this route. Larger droplets cannot penetrate the LRT	Virus is present and can survive on aerosols. Low titres of virus may be present but this may be compensated by the ability to penetrate the LRT where lower infectious doses are required. Few data are available to show viable virus exists on aerosols in natural conditions
Outbreak investigations	'Close contact' identified as important but difficult to distinguish between routes as all can act at short range	'Close contact' identified as important but difficult to distinguish between routes as all can act at short range		'Close contact' identified as important but difficult to distinguish between routes as all can act at short range. However, circumstances in three studies appear to support the existence of the aerosol route
Interventions	The HAND HYGIENE study by Talaat et al provides convincing data that this route of transmission is significant	SFMs show some effectiveness at reducing transmission but we are unable to say whether droplet, indirect contact or both are interrupted		Respirators appear no more effective than SFMs at reducing transmission suggesting a minor role for aerosol
Challenge studies	Virus has been recovered from fomites around experimentally infected volunteers but no studies have been done specifically assessing contact transmission	Infection can be initiated following direct nasal inoculation. The URT appears to need a higher infectious dose than the LRT. Nasally applied zanamivir does not prevent infection		Infection can be initiated by inhaling/respiring aerosols. The URT appears to need a higher infectious dose than the LRT. Inhaled zanamivir prevents experimental (intranasal) infection but not natural infection.
Animal studies	Does not appear significant but cannot infer that this is the case in humans	'Close contact' identified as important but difficult to distinguish between routes as all can act		Convincingly shown to be active in several studies
Modelling	Support for this route is apparent but relies heavily on assumptions	Support for this route is limited based on assumptions made that reaching target cells is problematic		Support for this route is apparent but relies heavily on assumptions



**If you or any of your family have flu
we need your help!**

Should you or other members of your family / household become unwell with symptoms such as cough, fever, sore throat, tiredness and runny nose over the next few weeks, we would like to invite you to take part in some medical research being run by the University of Nottingham.

The Department of Health has provided funding for this vital research. The study involves a nurse or doctor visiting daily to collect a nose swab and swabs from some surfaces in your home. Your help is really important to us. We hope to improve our understanding of how swine flu is spread which may lead to fewer people becoming infected.

So, if you or any family or household member develops flu-like symptoms and you/they feel able to take part in our study, please ring us and speak to one of our team. We are looking for people who have had symptoms for no more than 2 days so please call as soon as you think you are unwell. It does not matter whether medication is being taken or not.

Keep this card and call **0115 823 1813** anytime

Appendix 4.2: Eligibility criteria

DATE: ____ / ____ / 2009 Participant Code =

	Yes / Positive	No / Negative
Consent		
Symptoms;		
Fever		
Cough		
Sore throat		
Headache		
Fatigue		
Runny nose		
<ul style="list-style-type: none"> • Fever + 1 other <li style="padding-left: 20px;">or • 2 of the above 		
Symptoms for <48 hrs (Community)		
Symptoms for < 96 hrs (Hospital)		
Near Patient Test for influenza done?		
<ul style="list-style-type: none"> • If Yes, positive or negative? 		
Specific test for swine flu		
<ul style="list-style-type: none"> • If Yes, positive or negative? 		
Any other household member with symptoms?		
Taken part in other influenza research testing medicinal products in last 3 months?		

If only Green Boxes ticked = Eligible

Any Red boxes ticked = Not Eligible

Study title;

Virus shedding and environmental deposition of influenza virus

You are being invited to take part in this University of Nottingham sponsored medical research. Before you decide it is important for you to understand why the research is being done and what it will involve.

Please take time to read the following information carefully.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the research project?

There are some things that we do not know about how influenza is passed from person to person, for example how much virus exists on surfaces and how much is carried in the air. Such things are important to know because they might affect the advice that is given to healthcare workers about controlling the spread of infection to themselves and other patients. Similarly, this information is important for people who look after others in the home environment.

One way to try and gain information is to ask patients who get flu to help us by agreeing to give a daily nose swab sample for just over 1 week so we can see how much virus is in the nose day by day and how quickly this disappears. At the same time we will take samples from hard surfaces in a patient's room or home and sample the air using a special filter device. We can then work out how much virus is being released, how long the infectious period is and whether surfaces are more or less important than the air that we breathe (in terms of catching the virus).

The study involves a simple daily nasal swab and subjects who agree to take part will be inconvenienced to some extent. However, the technique of sampling from the nose is quick and not painful and should not present any problems. Normal medical care will not be affected in any way.

The team has been performing this kind of work for some time and is well qualified and experienced to carry out the study. Several members of the study team are leading international experts on influenza.

Why have I been chosen?

You have been chosen as you may have influenza.

This trial will include about 100 adults and children from Nottingham, Leicester and Sheffield. We are recruiting patients both from the community and in hospital.

Do I have to take part?

No. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time, without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive. If you do withdraw, we will ask why, as it might be important for other people, but you don't have to give a reason if you don't want to.

What will happen to me if we agree to take part?

If you choose to take part, the care you receive will not be different from that should you choose not to take part. You will be asked to sign a consent form. You will be given a copy of the information sheet and signed consent form to keep for your records.

We will confirm your entry into the study following a few questions. We will ask about your symptoms and their duration and if anyone else in your household has been ill. If your answers fit our criteria we will do two tests to try and confirm that you have influenza. Both tests involve a nose swab; one test will be done whilst we are with you. These tests are only being done for research purposes, though they might be helpful to your doctor. If so, we will let them know the results (with your consent).

If eligible, you will be involved in the trial for a maximum of 10 days and a minimum of 7. The number of days will depend on how long you have had symptoms before we meet you. If we meet on the day your symptoms begin we would like to visit every day for 10 days. If we meet 2 days after symptoms begin we will visit every day for 8 days. A member of the research team will carry out the visit; the person will usually be a nurse

but maybe another healthcare professional. All staff will have undergone the necessary checks and training needed to conduct such work. We will arrange appointment times with you.

We would like to visit you every day during the study and perform the following procedures (in addition to what has been mentioned above already);

- Symptom assessment – At the first visit you will be asked to complete a number of assessment forms that cover your medical history and current symptoms. Subsequently we will ask you to complete a diary of your symptoms. You will complete a simple chart which asks whether you are feeling certain symptoms and how severe they are. In addition to this we will take an oral temperature reading.
- Nose swab – A large cotton bud will be used to take a swab from the inside of the nose; it does not need to go very far back! This will be collected once every day (except on the first day when it might be done three times).
- Surface sampling – We have already chosen a number of common household and hospital room surfaces that we would like to swab, e.g. taps, door handles, remote control. We want to see if we can find influenza virus on these surfaces. We will take swabs every other day when we visit. You will be randomly split into two groups for this; Group 1 will have swabs done on Days 1, 3, 5, 7 and 9. Group 2 will be done on Days 2, 4, 6, 8 and 10.
- Air sampling – For a few patients we would like to conduct some air sampling in the room in which they spend most time. This involves running 2 small machines that suck in air and collect air particles. We want to see if we can find influenza virus in these particles. The machines will stand in a room and run for a maximum of 3 hours. They do make a small amount of noise. This will be done every other day during the study. A member of the research team will be present to set the machine up and collect it afterwards.

Each of the visits will last for up to 1 hour except when air sampling is performed (see above) which will take longer. The researcher may set up

the air sampling equipment, leave it running and then return before it finishes.

If you have been recruited in hospital and are later sent home, we would wish to follow you up at home for the remainder of the study period. Similarly, if you have been recruited in the community and need to be admitted to hospital we would follow you up in hospital.

This study will not interfere with the normal medical care you may receive. This includes the use of any medicines, e.g. antivirals

If for any reason you lose the capacity to consent during the study (e.g. the remote possibility that they are admitted to hospital and need to be sedated to help with breathing) we have included a box in the consent form to tick if you are happy for us to continue with our sampling during this period.

Initially your diagnosis of flu is likely to have been made on clinical grounds, i.e. the symptoms that you have. Doctors may have done a test to confirm this diagnosis (this may be different from the test we might have done initially on the nose swab). If flu is confirmed you will remain in the study but should this test come back negative for flu we will not perform any further sampling on or around you and you will be excluded from the study.

What are the possible benefits of taking part?

There is no specific treatment benefit as we will not influence your normal care. The work as a whole is seeking to provide information on influenza infection that could improve the way we deal with it, particularly from an infection control point of view and the public will benefit from this. You may gain some reassurance from the fact that a member of the research team will be visiting each day. However, as stated above they would not interfere directly with normal medical care. Of course, should there be any concerns they will raise them with you or your family so that you can contact your GP or other responsible medical professional.

Contact details

If you have any problems, concerns or other questions about this trial, you should contact the research member of staff who visits each day. If you have any complaints about the way the research staff are carrying out the study you can make a complaint to the study Chief Investigator, Professor Jonathan Van-Tam, Clinical Sciences Building, City Hospital, Hucknall Road, Nottingham, NG5 1PB. Tel 0115 823 0276.

What will happen if I don't want to carry on with the trial?

You can withdraw from the study at any time but it would be best to stay in contact with us and keep to the study assessments if possible. We will ask for your reasons for withdrawing, as they might be important for other people. You don't have to give any reasons if you don't want to.

What if there is a problem?

In the event that something goes wrong and you are harmed during the trial the University of Nottingham carries insurance to make sure that if any participant incurs any unexpected adverse event that leads to their being harmed and that the event occurred as a consequence of the protocol (i.e. non-negligent harm), then the participant will be compensated. In addition, all research staff have their own professional indemnity insurance which will cover any unexpected adverse event that leads to participant harm caused by negligence.

This study will be conducted in accordance with International Conference on Harmonisation (ICH) Good Clinical Practice (GCP) guidelines (directive CPMP/ICH/135/95), local regulatory requirements and the declaration of Helsinki, and all relevant local laws and regulations.

Will my participation in this trial be kept confidential?

When you enter the trial the researcher will record information about your illness, medical history and the subsequent course of the illness. Some of this information may be taken from your medical notes (if you are in hospital). Collection and analysis of this information is an important part of the research. Your contact details will also be recorded but will be kept separate from the study data on a secure database.

The results of the trial will be published in medical journals and sent to regulatory authorities. However, all identifying personal details will be kept strictly confidential and no information will be published or given out through which you could be identified.

What will happen to the results of the trial?

Any results will be presented to the Department of Health in the first instance. Subsequently, results may be presented at scientific medical meetings and published in a leading medical journal and possibly in national and local media too. You will not be individually identified in any report or publication.

Who is organising and funding the research?

The University of Nottingham is organising this study. The NHS Health Technology Assessment (HTA) Programme has provided the research grant and no member of the research team are being directly paid for including you in this study.

Who has reviewed the study?

The trial was peer reviewed before funding by the HTA. This study was given a favourable ethical opinion for conduct in the public-health sector by the Leicester Research Ethics Committee, and was approved by the local NHS Trust Research & Development departments.

You will be given a copy of this Adult Information Sheet and a copy of the signed Consent Form to keep.

THANK YOU FOR TAKING THE TIME TO READ THIS INFORMATION SHEET

Young Person Information Sheet (9-15 year olds)

A Study To Find Out How Much Flu Is Around You

What is research?

Research helps us to improve how much we know about things. This study is research to find out how much flu people carry around with them when they are ill.

Your invitation:

Would you like to be in this trial?

Before you decide, read this leaflet carefully. Talk about it with your family, friends, doctor or nurse.

Ask us if there is anything that's not clear or if you want to know more.

Why have I been asked to help?

Because you are unwell with flu. 50 children aged 0 to 16 years will be helping.

Do I have to take part?

No! It's up to you. If you do help, you can still pull out at any time. If you do decide to stop this won't upset anyone. If you do pull out, we will ask you why, as it might be important for other

young people. You don't have to give a reason if you don't want to.

What will happen to me?

We would like to take a sample from your nose using a cotton bud and we will take some samples from objects and even the air around you. When we take samples from your nose it won't hurt.



We will also ask you to answer some questions about how you are feeling each day and we will take your temperature.

We will visit you every day, for about 10 days. You may be in hospital or at home, we will follow you wherever you go!

You will be visited by a member of our team, usually a nurse. They will make appointments to see you and your parents.

Might anything else about the research upset me?

We don't think so!

Will joining in help me?

It won't help to make you better faster but the information we get might help us prevent other people from catching flu.

What happens when the trial stops?

Nothing! You should be feeling better and we have the samples we need.

What if something goes wrong?

Any trouble you or your parents have will be looked into. Details about this are in the Parent / Guardian Information Sheet.

Will my medical details be kept private? Will anyone else know?

Yes. Some people (called research inspectors) may see your medical notes to make sure the study is done properly.

What if I don't want to do the trial anymore?

You and your parents can pull out of the trial treatment at any time.

You will have a copy of this Information Sheet to keep.

THANKS FOR READING THIS – please ask us anything you want.

Contact details:

If you have any worries or questions, please tell your parents.

You can also contact;

Study Doctor:

Prof Jonathan Van-Tam

0115 823 0276

Child Information Sheet (0-8 year olds)

A Study To Find Out How Much Flu Is Around You

Your invitation:

Can you help us do this study?

Talk about it with your family, friends, doctor or nurse.

And ask us lots of questions!

Why have I been asked to help?

Because you are unwell with flu. 50 children aged 0 to 16 years will be helping.

Do I have to take part?

No! It's up to you. If you do help, you can change your mind later. This won't upset anyone.

What will happen to me?

We would like to take a sample from your nose using a cotton bud and we will take some samples from objects and even the air around you. When we take samples from your nose it won't hurt.

We will visit you every day, for about 10 days. You may be in hospital or at home, we will follow you wherever you go!



You will be visited by a member of our team, usually a nurse. They will make appointments to see you and your parents.

Will joining in help me?

It won't help to make you better faster but the information we get might help us prevent other people from catching flu.

What if something goes wrong?

Any trouble you or your parents have will be looked into. Details about this are in the Parent / Guardian Information Sheet.

Will my medical details be kept private? Will anyone else know?

Yes. Some people (called research inspectors) may see your medical notes to make sure the study is done properly.

What if I don't want to do the trial anymore?

You and your parents can pull out of the trial treatment at any time.

You will have a copy of this Information Sheet to keep

THANKS FOR READING THIS – please ask us anything you want.

Contact details:

If you have any worries or questions, please tell your parents. You can also contact;

Study Doctor: Prof Jonathan Van-Tam - 0115 823 0276.

Appendix 4.5: Symptom Diary Card

Subject Number <input type="text"/>		Subject Initials <input type="text"/>		Date <input type="text"/>	
Time of Day: ____:____ (24 hour)					
Day: <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10 <input type="checkbox"/> 11 <input type="checkbox"/> 12					
Place an "X" in the box in each symptom row that best describes how you have felt since completing your last diary card. Grade your symptoms based on the descriptions provided. Use the space to the right to note down any other symptoms you have.					
Level	0	1	2	3	Other Symptoms:
Symptoms:	I have NO symptoms	Just noticeable	It's clearly bothersome from time to time, but it doesn't stop me from participating in activities	It's quite bothersome most or all of the time, and it stops me from participating in activities	
Runny Nose					
Stuffy Nose					
Sneezing					
Sore Throat					
Earache					
Sinus Tenderness					
Malaise (tiredness)					
Cough					
Shortness of breath					
Diarrhoea					
Vomiting					
Headache					
Muscles and/or joint ache					

Appendix 4.6: Laboratory methods

PCR

Nucleic acid was extracted from the samples using the Qiagen Symphony SP extractor mini kits, including on-board lysis and a bacteriophage (MS2) internal control. A novel influenza A H1N1 pentaplex assay was devised to detect virus genome in the samples. The assay was designed to detect novel H1N1 influenza A, seasonal H1 influenza A, seasonal H3 influenza A, influenza B and the internal control, MS2. Reactions were carried out on a Rotorgene™ 6000 (Corbett Research) real-time DNA detection system. Viral load data were generated using the PCR assay and plasmids containing the gene target to create a standard curve, such that the concentration of genome present in each sample could be calculated.

The primers and probes used were as follows:

Primers

Novel H1N1 influenza A (Metabion):

- H1FORSW: 5'-TCA ACA GAC ACT GTA GAC ACA GTA CT-3'
- H1REVSW: 5'-GTT TCC CGT TAT GCT TGT CTT CTA G-3'

Seasonal H1 influenza A (MWG Biotech):

- AH1 Forward: 5'-GGA ATA GCC CCC CTA CAA TTG-3'
- AH1 Reverse: 5'-AAT TCG CAT TCT GGG TTT CCT A-3'

Seasonal H3 influenza A (MWG Biotech):

- AH3 Forward: 5'-CCT TTT TGT TGA ACG CAG CAA-3'
- AH3 Reverse: 5'-CGG ATG AGG CAA CTA GTG ACC TA-3'

Influenza B (Metabion):

- BNP-F: 5'-GCA GCT CTG ATG TCC ATC AAG CT-3'
- BNP-R: 5'-CAG CTT GCT TGC TTA RAG CAA TAG GTC T-3'

MS2 control (MWG Biotech):

- MS2 Forward: 5'-TGG CAC TAC CCC TCT CCG TAT TCA CG -3'
- MS2 Reverse: 5'-GTA CGG GCG ACC CCA CGA TGT=A C-3'

Probes

Novel H1N1 influenza A (Metabion):

- H1SWp3: 5'-Cy5-AAT GTA ACA GTA ACA CAC T CTG TTA ACC BHQ-3'

Seasonal H1 influenza A (ABI):

- AH1 Probe: 5'6FAM CGT TGC CGG ATG GA-MGBNFQ-3'

Seasonal H3 influenza A (ABI):

- AH3 Probe: 5'VIC-CCT ACA GCA ACT GTT ACC-MGBNFQ-3'

Influenza B (Biosearch Technologies):

- Flu-B Probe: 5'Quasar 705-CCA GAT CTG GTC ATT GGR GCC CAR AAC TG-BHQ-2-3'

MS2 control (Metabion):

- MS2 Probe: 5'ROX-CAC ATC GAT AGA TCA AGG TGC CTA CAA GC-BHQ-2-3'

RT-PCR protocol:

RT - PCR reactions comprised of 5µl of RNA and 20µl of mastermix (see table below). Primer probes were present at concentrations of 0.04 (AH1), 0.08 (Flu-B) and 0.08 (MS2) pmol/µl in the reaction mix. Cycles were performed as follows: reverse transcription at 50°C for 30 minutes, denaturation at 95°C for 2 minutes and then 50 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Stock concentration (pmol/μl)	Volume of stock/reaction (μl)
H1FORSW (20pmol/μl)	0.5
H1REVSW (20pmol/μl)	0.5
AH1 Forward (50pmol/μl)	0.45
AH1 Reverse (50pmol/μl)	0.45
AH3 Forward (50pmol/μl)	0.45
AH3 Reverse (50pmol/μl)	0.45
BNP-F (20pmol/μl)	0.25
BNP-R (20 pmol/μl)	0.25
MS2 Forward (20pmol/μl)	0.1
MS2 Reverse (20 pmol/μl)	0.1
H1SWp3 (10pmol/μl)	0.2
AH1 Probe (10pmol/μl)	0.1
AH3 Probe (10pmol/μl)	0.1
Flu-B Probe (10pmol/μl)	0.2
MS2 Probe (10pmol/μl)	0.2
2 x RT Platinum buffer (Invitrogen)	12.5
Superscript III Platinum enzyme	0.5
Water	2.7
Total volume:	20

Culture

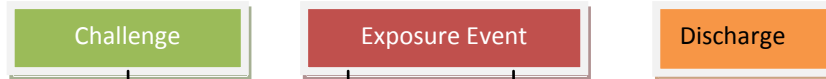
Influenza A(H1N1)pdm09 did not readily form plaques on MDCK cells so an immunofluorescence (IF) assay was used to detect the influenza A/B nucleoprotein in order to demonstrate the presence of live replicating virus in the swab samples. Assays were performed on samples that were PCR positive. On occasions if a swab was IF positive on a given day (e.g. Day 5) then an assumption was made that previous days (e.g. 1-4) would also have been positive and no testing on these days was done.

Madin Darby Canine Kidney (MDCK) cells were used to propagate the virus. Initially, cells were plated onto 6 well tissue culture dishes at a concentration of 7.5×10^5 /well; after 24 hours incubation the samples were defrosted. The cells were washed x2 in serum free medium (SFM, Dulbeccos Modified Eagles Medium, DMEM) and 400 μ l of each sample applied to the respective well. After 30 minutes the cells were overlaid with 2mL serum free medium containing 0.14% Foetal Calf Serum (FCS) and 0.1% Worthington's Trypsin. 1:10 dilutions of influenza A (H1N1 human influenza virus A/PuertoRico/8/34) and a novel H1N1 influenza A isolate (A H1N1 Cambridge AHO4/2009) were also inoculated onto cells as positive controls. The cells were then incubated for 48 hours at 37°C. The following day, 24-well tissue culture dishes were seeded with 1×10^5 MDCK cells per well; 48 hours later virus was harvested. Two dilutions were made in serum free medium, 1:2 and 1:10 (Yr1 only). After washing the cells in the 24-well dishes x 2 in SFM, 250 μ l of each dilution was added to the appropriate well.

Following 30 minutes incubation at 37°C, 1 mL of overlay (as before) was added to each well and the cells incubated overnight. After overnight incubation, the virus dilutions were aspirated off the cells. The cells were

washed x 2 with phosphate buffered saline (PBS) and then fixed with 250 µl of 4% formaldehyde at room temperature for 20 minutes. The fix was aspirated off and the cells washed x3 with blocking solution (1% FCS in PBS). The cells were permeabilised in detergent (0.2% Triton x100 in PBS) and then washed x2 in block solution. 250 µl of a mouse monoclonal antibody (for influenza A = Abcam ab43821, 1:1000 dilution Year1, 1:500 dilution Year2 ; influenza B = Abcam ab54142, 1:1000 dilution) was added to each well and the plates incubated 60 minutes before washing x3 with blocking solution. The secondary antibody (goat anti-mouse 488 IgG2a, Molecular Probes) was diluted 1:1000 in blocking solution, and 4, 6 diamino-2-phenylindole (DAPI) diluted 1:2000. 250 µl of this mix was added to the cells. Incubation was in the dark for 30 – 45 minutes. Cells were washed thoroughly with blocking solution, left in PBS and examined on the fluorescence microscope.

Appendix 5.1A: Study Schedule – Donors



STUDY DAYS Donors	Panel Screen	Study Specific Screen	Day -2 Quarantine start	Day -1	Day 0 Pre Influenza challenge	Day 0 Post Influenza challenge	1	2	3	4	5	6	(7)	Day 28 Follow up
Written consent	X	X												
Medical history /inclusion criteria	X	X	X											
Admit to Quarantine			X											
Influenza serology blood	X*	X*	X	(X)										X
Coagulation		X	(X)						X		X		(X)	X
Cardiac iso-enzymes		X	(X)						X		X		(X)	X
Interval medical history			X	(X)										
Directed physical exam			X	X	X	X**	X	X	X	X	X	X	(X)	
Complete physical exam		X	X	X								X	(X)	X
Symptom diary card			X	BD	X	X	BD	BD	BD	BD	BD	BD	(BD)	
Otосcopy			X	X	X	X	X	X	X	X	X	X	(X)	X
AEs + Con Meds			X	X	X	X	X	X	X	X	X	X	(X)	X
Nasal wash		X	X	(X)			X	X	X	X	X	X	(X)	X
Throat swab		X	X	(X)			X	X	X	X	X	X	(X)	X
Clinical *haematology & *biochemistry		X	(X)				FBC only		X		X		(X)	X
Dipstick urine		X	(X)						X		X		(X)	X
Pregnancy βHCG (urine)		X	X		X								(X)	X
HIV, Hep B & C screening		X												
Pulse/BP/RR		X	BD	QDS	X	TDS	QDS	QDS	QDS	QDS	QDS	QDS	(X)	X
Oral temperature		X	BD	QDS	X	TDS	QDS	QDS	QDS	QDS	QDS	QDS	(X)	
ECG		X	(X)				X	X	X	X	X	X	(X)	X
Spirometry		X	(X)		X		X	X	X	X	X	X	(X)	X
Class A drug screening		X	X	(X)	(X)		(X)	(X)	(X)	(X)	(X)	(X)		
Alcohol + Nicotine screening		X	X	(X)	(X)		(X)	(X)	(X)	(X)	(X)	(X)		
Oseltamivir administration										X	X	X	X	

Appendix 5.1B: Study Schedule - Recipients

STUDY DAYS RECIPIENTS	Panel Screen	Study Specific Screen	Day 0 Quarantine starts	Day 1	Day 2 Pre Influenza exposure	Exposure Event		4	5	6	7	8	9	10	(11)	Day 28 Follow up
						Day 2	3									
Written consent	X	X														
Medical history /inclusion criteria	X	X	X													
Admit to Quarantine			X													
Influenza serology blood	X*	X*	X	(X)												X
Coagulation		X	(X)				X		X		X		X		(X)	X
Cardiac iso-enzymes		X	(X)				X		X		X		X		(X)	X
Interval medical history			X	(X)												
Directed physical exam			X	X	X	X	X	X	X	X	X	X	X	X	(X)	
Complete physical exam		X	X	X										X	(X)	X
Symptom diary card			X	BD	X	X	BD	BD	BD	BD	BD	BD	BD	BD	(BD)	
Otoscopy			X	X	X	X	X	X	X	X	X	X	X	X	(X)	X
AEs + Con Meds			X	X	X	X	X	X	X	X	X	X	X	X	(X)	X
Nasal wash		X	X		(X)	X	X	X	X	X	X	X	X	X	(X)	X
Throat swab		X	X		(X)	X	X	X	X	X	X	X	X	X	(X)	X
Clinical *haematology & *biochemistry		X	(X)				FBC only		X		FBC only		X		(X)	X
Dipstick urine		X	(X)						X				X		(X)	X
Pregnancy βHCG (urine)		X	X		X										(X)	X
HIV, Hep B & C screening		X														
Pulse/BP/RR		X	BD	QDS	X	TDS	QDS	QDS	QDS	QDS	QDS	QDS	QDS	QDS	(X)	X
Oral temperature		X	BD	QDS	X	TDS	QDS	QDS	QDS	QDS	QDS	QDS	QDS	QDS	(X)	
ECG		X	(X)				X	X	X	X	X	X	X	X	(X)	X
Spirometry		X	(X)		X		X	X	X	X	X	X	X	X	(X)	
Class A drug screening		X	X	(X)	(X)		(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)		
Alcohol + Nicotine screening		X	X	(X)	(X)		(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)		
Oseltamivir administration												X	X	X	X	

Key: (X) = conducted at discretion of Study Physician; X = once daily; X* = conducted either at Panel screening &/or Study Specific screening visit; X** = 8 hours after challenge / exposure; QDS = 4 times a day; TDS = 3 times a day; BD = 2 times a day; FBC = Full Blood Count; *haematology & *biochemistry = abnormal results may be followed up by additional blood draws as deemed necessary by the CI

Appendix 5.2: Participant information sheet

Protocol title:	STUDY 1 Proof of concept: Confirming the Transmissibility of Experimentally Induced Influenza Infection Using an Approved GMP Challenge Virus
Protocol No.:	ITSDG 001, Final Version 4.0 20Jan2009
Protocol Sponsor:	University of Nottingham
Chief Investigator:	Professor Jonathan Van-Tam
Study Coordinating Site:	Retroscreen Virology Limited

Introduction

You have been invited to participate in a research study involving a virus that causes influenza in healthy adults. The virus is called Influenza A. Before agreeing to participate, it is important that you take time to read and understand this form. It describes why the study is being done, what will happen to you if you participate, what the risks or benefits may be, and what your rights are, whether or not you choose to participate. This study is carried out under the supervision of Professor Jonathan Van-Tam. Other professional persons (doctors, nurses, and technical persons) will assist him. If you have any questions, now or later, be sure to ask for an explanation.

Influenza A (H3N2) virus

The disease being studied is Influenza A (also known as the "flu"). Influenza is a viral disease of the respiratory tract. Typical influenza illness is characterised by an abrupt onset of fever, headache, myalgia (muscle aches), sore throat and cough. In healthy adults the illness usually resolves without any treatment, with relief of symptoms occurring naturally within 5 to 7 days.

Influenza virus is spread by inhaling droplets that have been coughed or sneezed out by an infected person or by having direct contact with an infected person's secretions from the nose. Handling household items or surfaces that have been contaminated by an infected person or an infected person's secretions may also spread the virus. Influenza causes annual

worldwide epidemics which in England typically occur from October to April. There is also currently concern about the possible emergence of pandemic influenza which would have a global impact. The virus infects all age groups; but the elderly, the weak or individuals with breathing difficulties or heart disease are at most danger. There are effective treatments and vaccines for Influenza. Approved treatments for Influenza include antiviral medications called Tamiflu (oseltamivir), Relenza (zanamivir) and amantadine. Retroscreen Virology has conducted Influenza studies in over 250 people to date in order to further study the Influenza virus and to conduct research into additional effective treatments and vaccines against 'Flu'.

What is the purpose of this research study?

The aim of this study is to find out whether the influenza virus we use to infect subjects is transmissible to other subjects. Some volunteers will be infected with the virus by instilling droplets in the nose (Donors). When Donors develop symptoms of flu, other volunteers (Recipients) will then be exposed to them by occupying the same room and taking part in certain procedures. If transmission of infection from Donors to Recipients can be demonstrated, it will allow for further studies looking at the mechanisms and routes of transmission and ways to reduce it.

What organisation reviews the study?

The Plymouth Independent Ethics Committee has reviewed the proposed research project and consent form and have given their favourable opinion for the project to take place.

What organisation is paying for the study?

This study is sponsored by the University of Nottingham and funded by the UK Department of Health. Retroscreen Virology Ltd will be carrying out the study.

Procedures during the study

- Up to 60 healthy adult volunteers will be entered into this study to ensure that a maximum of 24 healthy adult volunteers are available to enter a residential quarantine facility (an area separated from members of the public) and be given/exposed to the virus. Of these, up to 9 will be allocated to the 'Donor' group and up to 15 to the

'Recipient' group.

- Screening assessments before the quarantine stay will have been performed on two or three occasions.
- The duration of the quarantine stay will be approximately 8 days for Donors and 12 days for Recipients.
- At approximately 28 days after the start of the quarantine stay, you will be asked to return to the Retroscreen Virology clinic for a check up.

Assessments in this study will help ensure your safety. These include:

Medical History- Detailed questioning by the study doctor about your present and past medical history. It also includes any history of medication or drugs taken.

Physical Examination - a complete physical examination of the muscles, skin, heart function, lung, ears, nose, throat and eyes.

Blood Pressure Measurement - This provides information on the condition of the heart and blood vessels. Electric or manual blood pressure meters requiring an inflatable cuff to be placed around your arm will be used.

Lung Function Test (Spirometry) – This is a measurement of breathing capacity, important for assessing how well your lungs are working and making sure you do not have a respiratory condition. You will be asked to take the deepest breath you can, and then exhale into the sensor as hard as possible, for as long as possible.

Electrocardiogram (ECG) - This measures the electrical activity of the heart and aids the diagnosis of heart disease. 12 small self-adhesive electrodes will be attached to the skin of the arms, legs and chest. Areas such as the chest, where electrodes are placed, may need to be shaved. The ECG is painless and takes a few minutes to complete.

Blood Test (Venepuncture) - Blood is taken by the doctor or nurse and is usually from a vein around the inside elbow. A flexible tube (known as a cannula) may be left in the vein to reduce the number of times a needle has to be inserted.

HIV and hepatitis screening

We require that you have negative test results for Hepatitis B and C and HIV (human immunodeficiency virus, the virus that causes the acquired immunodeficiency syndrome [AIDS]) before you take part in a study. We know that having certain infections such as HIV, Hepatitis B and Hepatitis C can damage the body's immune system and make the individual more vulnerable to infections.

As the study involves challenging you with influenza, it is essential for safety reasons that we exclude conditions that might make your symptoms more severe. This is why we test all volunteers for HIV and Hepatitis B and C before they can take part. If you do not want to be tested for Hepatitis B, Hepatitis C or HIV then you should not agree to participate in this study.

A specific consent form for HIV testing can be found at the end of this form (page 14). You will not have to make a final decision about having this test until we call you back for your next visit (when the test will be taken); this could be several days to weeks from now but you will always have at least 24 hours from now in which to make a final decision. In the meantime you should read the further information on HIV below and the enclosed leaflet.

Further Information on HIV:

Many people throughout the world, including the UK are infected with HIV and do not know about it. One explanation for this is that the early stages HIV infection may not produce symptoms. It is for this reason that we perform the test, even if you do not consider yourself to be at risk (see below for at risk groups). The Retroscreen medical staff will answer any questions you may have about HIV/AIDS before your blood is taken for testing.

In the event of an unexpected positive test you will be informed and counselled by a study doctor. Arrangements can then be made (with your consent) to refer you to a specialist. Whilst HIV is not currently curable, effective medicines are available to keep the disease under control. You should be aware that any information given to us will remain completely confidential and this includes any test result. You should also be aware

that infection with HIV may have consequences for certain occupational groups and on the availability of health insurance/life assurance. Again, please free to discuss this further should you wish to.

Certain population groups have an increased risk of exposure to HIV and therefore may indicate a need for further counselling. These include:

- Gay or bisexual man.
- Current or ex-IV drug user.
- Unprotected sexual intercourse in a country of high HIV prevalence.
- A partner who has an increased risk of exposure to HIV.

Please inform a member of the medical staff if you are at increased risk or if your sexual history places you at an increased risk for HIV. They will then be able to answer any additional questions you may have.

Urinalysis- Examination of urine measures various compounds that pass through the kidneys. It includes

- Urine dipstick to pick up signs of infection or other conditions signifying ill health.
- Drug and nicotine testing.
- Pregnancy testing.

Nasal Wash- a procedure performed by the study doctor or nurse by pushing a water solution into the nose - one nostril at a time, so as to wash out and collect nasal secretions for analysis. It is performed at the screening visit and once daily on most days of the quarantine phase of the study.

Throat Swab – a small swab will be taken from the back of your throat.

Alcohol, drug and nicotine tests- performed randomly at the beginning and during the quarantine phase to help enforce a strict no alcohol, nicotine and drugs policy.

Study Schedule:

- **Study specific screening visit**

Up to 45 days before the quarantine start, you will be asked to sign this consent form. You cannot participate in this study if you were recently hospitalised, have recently taken antibiotics, have a longstanding medical

condition, have been enrolled in another medical study and have received an investigational medication within the last 6 months, have a blood test positive for Influenza A infection, a nasal wash positive for a respiratory virus, have ever received chemotherapy, or if you are a pregnant or breast-feeding woman. All sexually-active women must agree to use medically acceptable contraceptives throughout the study, up to and including Day 28 follow up. Medically acceptable contraceptives include: (1) surgical sterilisation, (2) approved hormonal contraceptives (such as birth control pills, Depo-Provera, or Lupron Depot), (3) barrier methods (such as a condom or diaphragm) used with a spermicide, or (4) an intrauterine device (IUD).

The following tests and procedures to make sure you are eligible to participate will take place:

- Medical history.
- Physical exam, to include heart rate, blood pressure, breathing rate and temperature
- Review of medications you may be taking.
- Blood sample (approximately 35mL, which is just over 3 tablespoons) for safety tests and to check your immunity to Influenza if this was not taken at pre-screening.
- Urine sample for safety tests and the screening of drug and nicotine abuse.
- Screening for alcohol.
- Nasal wash (two teaspoons [8mL] of sterile water will be placed into each nostril of your nose and be sucked out).
- A lung function test.
- Throat swab.
- An ECG (a tracing of your heart).
- Females only - a urine pregnancy test.
- Some blood taken will be used to screen you for HIV and hepatitis.

Participation

If all of the screening tests and assessments show that you are eligible, you may be asked to participate in the study. You may participate for approximately 3 months after the preliminary screening tests are complete. We will require a general practitioner's or doctor's referral form from your GP/Doctor stating his or her opinion on your suitability for

participation in the study. The GP/Doctor may charge a small fee (usually £30-£50) for this documentation, which we will reimburse to you. This information will be handled in a confidential manner.

- **Quarantine phase of the Study**

Donors will be admitted 2 days before the virus is administered (Study Day -2) whilst Recipients will be admitted 2 days later (Study Day 0).

To be admitted to the quarantine isolation facility you must agree not to smoke and not to consume any alcohol during this 8 to 12 day quarantine period. Using recreational drugs is also forbidden. Your belongings will be checked to ensure forbidden substances are not brought into the Quarantine Unit. We will conduct random alcohol, drug and nicotine testing. If you have a fever or symptoms suggesting a “cold or flu-like” infection between your admission and the following morning in the isolation facility, you will be sent home. The study doctor will provide a referral for medical care if necessary.

- In the Retroscreen Quarantine Unit you will be placed into “droplet precautions”. This means visitors to your room will be limited — anybody visiting you will wear a gown and mask before entering your room. All staff will be required to disinfect their hands thoroughly before and after entering your room. You will remain in your room, or areas designated for your use or recreation by the study staff, throughout your stay.
- Study diary: You will be asked to record your symptoms in a symptom diary card twice daily which will be collected by study staff daily.
- A cannula (a very small plastic tube that remains in your arm) may be placed in your hand or arm to allow for easy sampling of your blood. Alternatively, blood will be taken by standard blood sampling methods using a sterile needle.

The following assessments will take place on admission to the quarantine unit:

- Medical history and review to ensure you can continue in the study.
- Complete and directed physical examination including an ear examination.
- Temperature, heart rate, blood pressure, respiratory (breathing) rate monitored twice daily.
- Review of medications you may be taking.

- ECG.
- Lung function test.
- Blood sample (approximately 35mL) for antibodies and safety tests.
- Nasal wash.
- Throat swab sample.
- Drugs of abuse, alcohol and nicotine tests.
- Urine sample for safety tests.
- Females only - a urine pregnancy test.

If any of the above assessments need to be repeated, they will be done on the next day.

The following assessments will take place on the day after admission:

- Medical history and review to ensure you can continue in the study.
- Complete and directed physical examination, including an ear examination.
- Temperature, heart rate, blood pressure, respiratory (breathing) rate monitored twice daily.
- Review of medications you may be taking.
- A throat swab.
- A lung function test.
- Your symptom diary card will be checked – twice daily.

Donors Only - Administration of Influenza A virus (Day 0)

In the morning:

- Medical history and review to ensure you can continue in the study.
- Your pulse, blood pressure, breathing rate and temperature will be checked four times daily.
- Review of medications you may be taking.
- A lung function test.
- A direct physical examination and ear examination.
- Your symptom diary card will be checked by a study doctor.
- Urine samples will be collected for safety.
- Females only - a urine pregnancy test.
- Drug, alcohol abuse may be tested.

Exposure to virus:

- You will be asked to lie flat on your back.

- The study physician will place a few drops of a liquid solution into each of your nostrils.
- After receiving the nose drops, you will be asked to continue to lie flat on your back for about 15 minutes.
- You can then sit up after 15 minutes have passed.
- The study clinical team must monitor you for 15 more minutes.

Later in the afternoon / evening, you will have:

- A direct physical examination and an ear examination.
- A throat swab.
- Your symptom diary card will be checked by the study doctor.

Recipients Only – Exposure to Influenza A virus (Day 2)

In the morning:

- Medical history and review to ensure you can continue in the study.
- Your pulse, blood pressure, breathing rate and temperature will be checked four times daily.
- Review of medications you may be taking.
- A lung function test.
- A direct physical examination and ear examination.
- Your symptom diary card will be checked by a study doctor.
- Urine samples will be collected for safety.
- Females only - a urine pregnancy test.
- Drugs, nicotine and alcohol may be tested for.

Exposure Event (Day 2 – 4); Involves Donors with Symptoms and Recipients

The Exposure Event is a 48 hour period where volunteers (two Donors and five Recipients) will share living quarters, giving the opportunity for the virus to be passed from person to person. Each Event will start when two Donors develop symptoms of Flu. They will be joined by 5 Recipients making a total of 7 subjects per event.

During the day, subjects will be asked to perform set tasks including;

- Donor reading to a Recipient for 20 minutes.
- Donors and Recipients playing cards / games.
- Donors and Recipients eating meals together.

At night, some Recipients will sleep in the same room as a Donor.

Donors who do not develop symptoms

They will not take part in the Exposure Event but will remain in the quarantine unit and undergo the same tests and procedures as Donors with symptoms of flu (e.g. fever, cough, runny nose, sore throat).

Procedures to be performed 1 day prior to and during the Exposure Event and the during remaining days in quarantine

- You will continue to fill out your symptom diary cards twice daily.
- The study doctor will ask you about any signs or symptoms you may be experiencing, measure your temperature, and examine your eyes, ears, nose, throat, and lungs.
- Blood will be drawn on alternate days from Study Day 1-9 via a cannula or by a needle and syringe. On each occasion approximately 5-20mL of blood (1-2 tablespoons) will be drawn from you.
- An ECG and a test to monitor lung function will be performed daily.
- You will undergo a daily physical examination.
- Nasal wash & throat swab samples will be collected once a day.
- A urine specimen will be collected on two occasions (males) and three occasions (females).
- Random alcohol, drugs and nicotine tests may be conducted.

Antiviral medication

At a specified time point after you have been either challenged (Donor) or exposed (Recipient) to influenza virus you will be given an antiviral medication. The primary aim of this is to ensure that the virus is not spread to others when you leave the quarantine unit. However, this medication may also be used to treat signs and symptoms of influenza at the discretion of the study doctor.

Environmental Sampling

During the quarantine period we will be collecting samples from the environment. This will involve swabs taken from surfaces and air samples collected from an air sampling device. Some volunteers will be asked to carry a portable sampling device for a short period of time during the quarantine period.

Photography

During the quarantine stay we will take a limited number of photographs in order that we may pictorially describe the set up of the unit and in particular demonstrate the arrangements for close living quarters that will occur during the Exposure Event. We may use these photographs in presentations and/or publications about the study. No volunteer will be identifiable in any photographs used. Consent will be obtained from you so that we may take photographs.

Discharge from quarantine

If you appear free of viral infection at Day 6 (Donors) or Day 10 (Recipients) you will be permitted to leave quarantine. If some infection remains, you will be required to stay in quarantine for a further 24 hours or longer if required.

Follow-up visit (\approx Day 28)

You will return to the study doctor at the clinic

- A complete physical examination will be performed.
- Review of medications you may be taking will be recorded.
- Approximately 50mL (just under 4 tablespoons) of blood will be drawn for antibody & safety tests.
- A nasal wash will be performed.
- An ECG will be performed.
- A urine sample will be collected for safety tests.
- Females only - a urine pregnancy test will be conducted.

Storing and using samples and information from this study

- Some of the samples and the information collected during this study will be kept by Retroscreen Virology Ltd or sent to the University of Nottingham for research analysis.
- The University of Nottingham and Retroscreen Virology Ltd will store any samples and information in a secure place.
- The samples will be identified by a unique and anonymous study number, which means that your name and other identifying information will not be on the sample. The University of Nottingham will not receive your name or other information about your identity.

Research Results

The study described is for research purposes only. Therefore, you will receive no results from this study.

Risks for loss of confidentiality

Maintaining confidentiality is important to the Retroscreen Virology Limited and the University of Nottingham. They will keep all your samples and information confidential and secure. In addition the University of Nottingham will receive only your unique study number, not your name or other identifying information. Retroscreen Virology will not release your identifying information; therefore there will be no loss of confidentiality from information kept at the University of Nottingham.

Potential Risks

If you are chosen to participate in the study, you might experience symptoms of infection with Influenza A virus.

- Donors have about a 90% chance of becoming infected with influenza following the administration of the virus. We don't know how many Recipients will become infected. Influenza usually resolves without treatment and symptoms will normally go away after approximately 5-7 days.
- Influenza (flu) causes an infection of the lungs and airways. It causes a fever, runny nose, sore throat, cough, headache, muscle aches, and a general feeling of illness (malaise). Severe complications of Influenza tend to occur almost exclusively in children aged <12 months, the elderly, and persons of any age with chronic illnesses and with weak immune systems. Nevertheless, severe influenza infections are reported around the globe most years albeit in low numbers, amongst healthy adults. Occasionally, flu can progress to pneumonia, either caused by the virus itself or by a secondary bacterial infection. Qualified doctors and nurses will be in the quarantine unit with you at all times. Should you experience any symptoms, they will assess you and manage these symptoms.
- Starting on evening of Day 4 (Donors) or Day 8 (Recipients) of the quarantine stay, you will be administered a course of an antiviral medicine called oseltamivir. This is an approved treatment for Influenza that can lessen the degree and duration of symptoms. You

will be required to take oseltamivir to prevent the possibility of re-infection of influenza and also to prevent transmission of influenza to others when you leave the unit.

- Women will have a pregnancy test performed before receiving virus. Caution should be taken to avoid all infections during pregnancy. Therefore, any woman with a positive pregnancy test will not receive virus, and all women participating in this study must use a reliable birth control method consistently, to avoid becoming pregnant for the duration of the study. If you do become pregnant during this study, you must inform your study doctor immediately.
- It is unlikely that you will transmit Influenza to your close contacts when you leave the quarantine unit. After infection with Influenza A, the virus will be present in your nose for several days. It is not expected that enough Influenza A will still be in your nose for you to transmit to others once you leave the isolation facility. This is because the usual duration of time that Influenza remains in adults is several days shorter than the time you will spend in quarantine in the isolation facility. Your secretions from your nose will also be tested for Influenza prior to discharge and you will only be discharged if there is no detectable Influenza present.

To further reduce the risk of passing Influenza to others you should avoid contact with the following groups of people for 2 weeks after you leave the isolation facility:

- Children less than 3 years of age.
- Anyone with immune system problems or who has undergone an organ transplant.
- Anyone being treated for or about to be treated for cancer.
- Anyone with lung disease like emphysema.
- Elderly persons.

If you are a healthcare worker, nurse, doctor or medical student, you should not work with patients until 14 days after you have been challenged/exposed to the virus, or until your symptoms are fully resolved (whichever is the longer period).

A similar type of Influenza has been given to over 200 people by Retroscreen Virology Limited.

- The throat swab and nasal wash procedures may be mildly uncomfortable and you may gag or cough, or your nose may sting a little for a short time.
- Blood drawing causes momentary pain, sometimes a bruise, occasional lightheadedness and rarely fainting.
- Placement of an intravenous cannula (small plastic tube into the vein) is a routine procedure that involves momentary discomfort at the time of insertion. Minor bruising may occur. In rare cases, phlebitis (irritation of the vein), extravasation (leaking of fluid outside the vein) and infection may occur.
- The blood tests performed to assess your health may indicate that you have an infection you were not aware of (such as the HIV or hepatitis) or an unexpected illness.
- You will be told of any significant findings that develop during the course of this study that may influence your willingness to continue your participation.

If you have private medical insurance or life insurance, you should check that your participation in this study does not affect your insurance.

Participation in multiple research studies

You must not take part in too many or multiple studies because this may cause serious risk to your health. To avoid this, UK study units like ours keep a linked database of healthy volunteers and when volunteers take part in studies. We will enter the following details into the national database:

- your National Insurance number (if you're a UK citizen); or
- your passport number and country of origin (if you're not a UK citizen);
and
- the date of your last dose of study medicine/virus.

If you withdraw from the study before you receive any study medicine/virus, the database will show that you did not receive a 'dose'. Only staff at the Retroscreen Virology Clinic and other medicines research units can use the database. We may call other units, or they may call us, to check your details. We will check your information on the national database before you are admitted to the study.

Your details will be kept on the national database for at least 2 years. If we need to contact you about the study after you have finished it, but we can't because you have moved or lost contact with your GP, we might be able to trace you through the information in the database.

Potential benefit of study participation

It is not anticipated that any participant will derive personal benefit; however by taking part in this study you may contribute new information that may benefit patients in the future.

Alternatives to Participation

The alternative to participating in this study is to simply not to participate.

Compensation for your involvement

- During the isolation period of the study, if you are withdrawn either before the virus is administered (Donors) or before you are exposed to virus (Recipients), you will be compensated for your preceding visits and the nights spent at the isolation facility. This compensation will be £250.00 per night to a maximum of £500 and payable by BACS/cheque on your final visit.
- If you complete the full study;
 - Donor: You will be compensated £1800 payable by BACS/cheque in installments; 1st payment of £1,000.00 on your final day at the quarantine unit, with the remaining £800.00 on your Day 28 final visit at the end of the study.
 - Recipient: You will be compensated £2100 payable by BACS/cheque in installments; 1st payment of £1,000.00 on your final day at the quarantine unit, with the remaining £1,100.00 on your Day 28 final visit at the end of the study.
- If you are withdrawn from the study because of positive tests on alcohol, nicotine or drug screening, you will forfeit any compensation.
- In order to receive full compensation, you are expected to be available for all visits, the Quarantine Phase and the final follow up visit.
- If you believe you will be unable to attend all the required visits, then you should not participate in the study. If you fail to attend all scheduled clinic visits, your compensation will be reduced.
- You may be issued with FluCamp.com kit on completion of the

Quarantine phase – a FluCamp T-shirt, baseball cap, notepad, pen and water bottle may be given.

Confidentiality

Your name and identity will not be disclosed in any publications or reports resulting from this study. Professional employees of Retroscreen Virology Limited and the University of Nottingham will have supervised access to your study records. By signing this consent document, you agree to such inspection. These persons will use your study records only in connection with carrying out their obligations relating to the study. Your records will be kept as confidential as possible, within the limits of UK common law. How the information about you and your medical condition is stored and who has access to it, is governed by data protection laws (The Data Protection Act 1998).

Data from this study, in which your name and identity will not be disclosed, will also be transferred outside of the European Economic Area (EEA). Some non-EEA countries may not offer the same level of privacy protection as in the UK. The data will be anonymised before it is sent to these parties and therefore your personal information will be confidential at all times.

Right to withdraw from study

- Your participation in this study is entirely voluntary. You may refuse to participate in this study, or withdraw from this study at any time, without a penalty or loss of benefits to which you would normally be entitled.
- If you withdraw from the study, any stored blood or tissue samples that can still be directly identified as yours will be destroyed if you so request. No further data relating to you will be collected. We will continue to use the data collected up to the point of your withdrawal for the purposes of this study only.
- You must be aware that withdrawal after you have entered the isolation facility and received the Influenza A virus may result in risks to both your own health and that of people with whom you come in contact. You are very strongly advised to complete the full post-challenge/exposure observation period.
- In addition to providing study information, this observation period is

designed to detect any complications of Influenza early, so that you may receive treatment if needed.

- The Influenza virus that you will receive is likely to be capable of being spread to other persons and causing illness. Accordingly, should you choose to withdraw after receiving the virus, but before the observation period is complete, you will be encouraged to remain in the quarantine unit as long as possible and if released early, you will be encouraged to keep in contact with us to let us know your progress.

Involuntary withdrawal

Your participation in this study may be stopped without your consent if:

- You fail to comply with the requirements of the study.
- In the opinion of a study doctor, continued participation poses a risk to your health or well-being.
- The study is discontinued by Retroscreen Virology Limited, the University of Nottingham or the Research Ethics Committee.
- Retroscreen Virology Limited reserves the right to withdraw any volunteer from the study during the quarantine period if that volunteer either: a) has symptoms of an illness before receiving the virus or b) behaves in a manner that is dangerous to his/her health, dangerous to the health of others, or disruptive to the proper conduct of the study.
- Retroscreen Virology Limited staff will be the sole judge of inappropriate or dangerous behaviour.

Freedom of information

You are entitled to inspect your personal information stored by Retroscreen Virology Limited and are entitled to copies of such information. Copies will be provided within 40 days of a written request in accordance with The Data Protection Act 1998.

Contact for medical concerns or questions

If you have any questions about this study, if you experience any symptoms which you think might be related to participating in this study, or if you have any other medical problems, be sure to report them promptly to Dr. Anthony Gilbert (Senior Investigator at Retroscreen Virology) on 020 7756 1300 during office hours or call the 24 hour number which will be given to you for your use during this study.

If you have questions about your rights as a research subject volunteer or if you have questions, concerns or complaints about the research, you may contact:

Plymouth Independent Ethics Committee
Tamar Science Park
1 Davy Road
Derriford
Plymouth
PL6 8BX

Compensation for injury

The University of Nottingham carries insurance to make sure that if any participant incurs any unexpected adverse event that leads to their being harmed and that the event occurred as a consequence of the protocol (i.e. non-negligent harm), then the participant will be compensated. In addition, all study doctors have their own professional indemnity insurance which will cover any unexpected adverse event that leads to participant harm caused by negligence.

This study will be conducted in accordance with International Conference on Harmonisation (ICH) Good Clinical Practice (GCP) guidelines (directive CPMP/ICH/135/95), local regulatory requirements and the declaration of Helsinki, and all relevant local laws and regulations.

Yours sincerely,



Professor Jonathan Van-Tam
Chief Investigator

HIV testing consent form

Protocol title: STUDY 1 Proof of concept: Confirming the Transmissibility of Experimentally Induced Influenza Infection Using an Approved GMP Challenge Virus
Protocol No.: ITSDG 001, Final Version 1.0 23DEC2008
Protocol Sponsor: University of Nottingham
Chief Investigator: Professor Jonathan Van-Tam
Study coordinating site: Retroscreen Virology Limited

Consent to test	YES	NO
Have you been previously tested for HIV?		
Have you been given more than 24 hours to consider the information provided?		
Do you consent to a blood test for HIV?		

Points covered in pre-test discussion:	Tick if discussed
Risk Assessment – Unprotected sexual intercourse (number of partners since last test)	
Health implications of positive result	
Explain testing process & window period – need to wait or re-test after 3 months	
Confidentiality maintained	
Consequences of HIV testing – life assurance, employment etc.	

x

Signature of Volunteer

Date

x

Printed Name of Volunteer

x

Signature of Study Nurse/Doctor Obtaining Consent

Date

x

Printed name of Study Nurse/Doctor

Appendix 5.4: Exposure Event Schedule

3 Events will happen simultaneously (Groups 1-3)
Times are approximate

Day 2;

09.00	Symptomatic Donors (D1 & D2) and Recipients (R1 - R5) meet in designated quarantine area Each to spend minimum 30 minutes on computer each day (email / game)
10.30	D1 reads to R1 (20 minutes) D2 reads to R4 (20 minutes)
13.00 – 13.45	Sit down Lunch
15.00	D1 reads to R2 (20 minutes) D2 reads to R5 (20 minutes)
16.00 – 17.30	All play cards / monopoly at table
18.00 – 19.15	Sit down Dinner
19.30	D1 reads to R3 (20 minutes)
20.00 – 20.30	All play game e.g. charades
21.00	All watch film
23.00	END

Day 3;

08.00	Sit down breakfast Each to spend minimum 30 minutes on computer each day (email / game)
10.30	D1 reads to R4 (20 minutes) D2 reads to R1 (20 minutes)
13.00 – 13.45	Sit down Lunch
15.00	D1 reads to R5 (20 minutes) D2 reads to R2 (20 minutes)
16.30 – 17.30	All play cards / monopoly at table
18.00 – 19.15	Sit down Dinner
19.30	D1 reads to R3 (20 minutes)
20.00 – 20.30	All play game e.g. charades
21.00	All watch film
23.00	END

Appendix 5.5: Directed Physical Examination Worksheet

Subject Study ID Number: _____ Subject Initials: _____

Quarantine Phase Day (circle one):

-2 -1 0 1 2 3 4 5 6 7 8 9 10 11

Date: _____(day) _____(month) _____(year)

Time: (24 hour clock): _____ Initials: _____

	LEVEL 0	LEVEL 1	LEVEL 2	LEVEL 3
Upper Respiratory:				
Nasal discharge	None	Clear, serous; scant but slightly increased	Clear to white, obvious increased volume, ± minor blood streaks on tissue	Frankly purulent (yellow or green), or gross blood
Otitis	None	Dulled tympanic membrane	Inflamed, injected tympanic membrane	Retracted or bulging tympanic membrane, obvious air-fluid level
Pharyngitis	None	Mild and / or patchy erythema	Marked and /or confluent erythema	Erythema and purulent exudate
Sinus tenderness	None	NO LEVEL 1	Mild tenderness	Severe tenderness or overlying erythema
Lower Respiratory:				
New wheezes, râles, rhonchi, other	None	NO LEVEL 1	Scattered wheezes or rhonchi	Widespread wheezes or rhonchi; râles, dyspnoea, or signs of consolidation

Appendix 5.6: Laboratory methods

Challenge Virus Titre Determination

Immediately post preparation of the individual inocula the virus was back titrated on MDCK cells. The virus was titrated in quadruplicate from an initial dilution of 1/10 (v/v) (11 μ L in first row) following a 1/10 (v/v) titration series (11 μ L titrated across into 100 μ L MDCK infection media). Both the neat stock virus, as well as an aliquot of the diluted virus, were back-titrated at this point. At the time that the first volunteer was infected, as notified by clinical staff, a second vial of the diluted individual inocula was titrated as described above. The virus was back-titrated a third time at the time that the last volunteer was infected.

- The titre of the virus immediately post preparation was calculated to be 5.75 log₁₀TCID₅₀/mL.
- The titre of virus at the time that the first volunteer was infected was calculated to be 6.00 log₁₀TCID₅₀/mL.
- The titre of virus at the time that the last subject was infected was calculated to be 5.27 log₁₀TCID₅₀/mL.
- The geometric mean of the virus titres at the time of the first and last subject infection was calculated to be 5.64 log₁₀TCID₅₀/mL.

Retroscreen Haemagglutination Inhibition Assay

The serum from each sample was separated into 3 aliquots and stored at -20°C until required for analysis.

Prior to commencing the assay, the following procedures were performed:

- Challenge virus was diluted to 8 haemagglutination units (HAU).
- Positive control antiserum (anti A/Wisconsin/67/2005: NIBSC; 05/236) was diluted to 40 haemagglutination inhibition units (HIU).

- Serum samples were treated with Receptor Destroying Enzyme (RDE) to remove any non-specific inhibitors of the influenza virus that may cause non-specific agglutination.
- The HAI assay was performed as follows:
 - Serum samples and the positive control serum were titrated in PBS on V-bottomed 96-well plates following a 1/2 (v/v) dilution series.
 - An equal volume of challenge virus was added to each well and the plate incubated at room temperature for 30 minutes.
 - Turkey red blood cells were added to each well and the plate incubated at room temperature for 30 minutes.
 - The plates were read by eye to determine the presence or absence of agglutination for each sample.

Controls used in the assay were i) positive control antiserum, ii) negative control challenge virus (A/Wisconsin/67/2005: RVL; AL 1764) and iii) PBS control. The assay was performed in duplicate for each sample. The duplicate endpoints for each sample were all within one two-fold (v/v) dilution of each other. Where the duplicate endpoints were not identical, but within one two-fold (v/v) dilution of each other, the geometric mean of each endpoint was calculated.

CDC Serology Methods

Sera were tested by the hemagglutination inhibition (HAI) and/or microneutralization (MN) assays according to previously published procedures (Kendal *et al*, 1982; Rowe *et al*, 1999). The following viruses were used: A/Wisconsin/67/2005 (H3N2), grown in Madin-Darby canine kidney cells; A/Brisbane/10/2007 (H3N2), A/Brisbane/59/2007 (H1N1) and A/Mexico/4108/2009 (H1N1pdm), all grown in 10 to 11 day old embryonated chicken eggs. HAI testing was performed using 0.5% turkey

erythrocytes. For the MN assay, sera were heat inactivated at 56°C for 30 minutes prior to testing. For the HAI assay, sera were treated with RDE (Denke-Seiken, Japan) followed by heat inactivation at 56°C for 30 minutes and sera containing nonspecific agglutinins were pre-adsorbed with turkey erythrocytes prior to testing. For both assays, serial two fold dilutions of serum (1:10 to 1:1280) were tested in duplicate. HAI or MN titres were expressed as the reciprocal of the highest dilution of serum that gave complete hemagglutination or 50% neutralization, respectively.

Nasal Wash and Throat Swab Sample Processing

Samples were transported to the analytical laboratory on wet ice with the exception of the Day 3 and Day 4 samples;

- Day 3 samples were frozen on dry ice immediately after collection at the quarantine site and transported to the analytical laboratory on dry ice.
- Day 4 samples were processed directly at the quarantine site and the aliquots placed onto dry ice for transport to the laboratory and stored in a freezer set at -80°C upon arrival until required for analysis.

Nasal wash samples were aliquoted into 6 cryovials. The cryovial aliquots and the sample collection vessel were kept on wet ice throughout the procedure. Aliquots 1, 2 and 3 were aliquoted directly into cryovials. Each aliquot contained approximately 2mL. These aliquots were designated for qPCR analysis. Aliquots 4, 5 and 6 were aliquoted by transferring 500µL sample into a cryovial containing 500µL 50% (w/v) sucrose solution such that the final concentration of sucrose in the sample is 25% (w/v). The 50% (w/v) sucrose solution was prepared by dissolving sucrose in PBS [Gibco; 10010] and sterilizing by filtration prior to use. These aliquots were designated for the infectivity assay.

Throat swabs were taken out of the collection tube and rotated in the bijou containing a 4mL 1% (v/v) penicillin streptomycin solution. Prior to removal of the swab from the bijou, the swab was pressed against the side of the vial and turned to ensure excess liquid was removed. Samples were aliquoted into 3 x ~1mL aliquots in cryovials. The cryovials and the bijou were placed on wet ice throughout the procedure.

All aliquots were placed into a monitored freezer set at -80°C until required.

Retroscreen Influenza Infectivity Assay

MDCK cells were seeded at a density of $\sim 5 \times 10^4$ cells/mL into 96-well plates the day prior to use in the assay. On the day of the assay the cell monolayers were washed twice with 100 μ L of PBS and 100 μ L of MDCK infection media was added to each well. The nasal wash and throat swab samples were thawed and vortexed. 11 μ L of the sample was added in quadruplicate to the first row of wells on the plate. A suitable control virus was used as a titration control in quadruplicate on every plate. The samples and control virus were titrated following a 1/10 (v/v) titration series (11 μ L titrated across into 100 μ L MDCK infection media). The last row of wells was used as the cell only control. The plates were incubated in at 37°C ($\pm 2^\circ$ C); 5% CO₂ for 3 days and the end point was determined by haemagglutination assay. After the 3 day incubation period 50 μ L of the supernatant from each well on the 96-well titration plate was transferred to the corresponding well on a fresh v-bottomed 96-well assay plate. 50 μ L of 0.5% (v/v) turkey red blood cells in PBS was then added to each well on the v-bottomed assay plate to give a total volume of 100 μ L. The plates were incubated at room temperature for approximately 30 minutes before being read visually for the presence of virus in each well. Each well was

scored positive or negative. Haemagglutination of the red blood cells indicated a positive result and sedimentation of the red blood cells into the apex of v-shaped well indicated a negative result. The qualitative haemagglutination results were used to calculate the virus titre using the Karber Calculation.

PCR protocols

- HPA Laboratory Cambridge; Influenza – National Standard Method VSOP 25
- HPA Laboratory Cambridge; Respiratory virus PCR protocol - VSOP 086:
The virus panel includes; Influenza A + B, parainfluenza, rhinovirus, adenovirus, metapneumovirus, coronavirus and RSV.
- Lab 21 Healthcare; Influenza A:
Primer sequences were initially obtained unmodified. Since 2009 modified versions of these primers, in which mismatches to pandemic H1N1 strains have been corrected, have been added to the assay. The assay is a nested PCR and is interpreted by electrophoresis and UV transillumination in the presence of ethidium bromide.

Appendix 5.7: Influenza PCR results on nasal wash and throat swab samples

		Study Day						
		3		4		5	6	7
Laboratory →	Specimen →	HPA NW	Lab 21 TS	HPA NW	Lab 21 NW	HPA NW	HPA NW	HPA NW
D01		+	-	+	+	+	+	ND
D02		+	+	+	+	+	+	ND
D03		+	-	+	+	-	-	ND
D04		-	-	-	-	-	-	ND
D05		+	+	+	+	+	+	ND
D06		-	-	-	-	-	-	ND
D07		+	-	+	+	+	-	ND
D08		-	-	-	-	+	+	ND
D09		-	+	-	+	-	-	ND
R01		ND	-	-	-	-	-	-
R02		ND	-	-	-	-	-	-
R03		ND	-	-	-	-	-	-
R04		ND	-	-	-	-	-	-
R05		ND	-	-	-	-	-	-
R06		ND	-	-	-	-	-	-
R07		ND	-	-	-	-	-	-
R08		ND	-	+	-	-	-	-
R09		ND	-	-	-	-	-	-
R10		ND	-	-	-	-	-	-
R11		ND	-	-	-	-	-	-
R12		ND	-	-	-	-	-	-
R13		ND	-	-	-	-	-	-
R14		ND	-	-	-	-	-	-
R15		ND	-	-	-	-	+	-

Footnote: HPA = Health Protection Agency laboratory, Lab 21 = Laboratory 21, Cambridge, NW = Nasal Wash, TS = Throat Swab, + = Positive, - = Negative, ND = Not Done

Appendix 5.8: Retroscreen virology Ltd influenza serology (HAI) results

Study Code	Subject Number	Subject Initials	Sample Type	Study Day	Collection Date	HAI titre	Virus Strain
ITSDG-001	106112	R-R	SERUM	0	31-May-09	NDA	A/Wisconsin/67/2005
ITSDG-001	102137	H-J	SERUM	0	31-May-09	NDA	A/Wisconsin/67/2005
ITSDG-001	101550	MPR	SERUM	0	31-May-09	NDA	A/Wisconsin/67/2005
ITSDG-001	106120	FLA	SERUM	0	31-May-09	NDA	A/Wisconsin/67/2005
ITSDG-001	105804	RJG	SERUM	0	31-May-09	NDA	A/Wisconsin/67/2005
ITSDG-001	101571	PNR	SERUM	0	31-May-09	<20	A/Wisconsin/67/2005
ITSDG-001	105872	DRT	SERUM	0	31-May-09	NDA	A/Wisconsin/67/2005
ITSDG-001	106057	MTC	SERUM	0	31-May-09	NDA	A/Wisconsin/67/2005
ITSDG-001	104320	G-T	SERUM	0	31-May-09	NDA	A/Wisconsin/67/2005
ITSDG-001	105771	ESK	SERUM	0	31-May-09	20	A/Wisconsin/67/2005
ITSDG-001	103752	TSG	SERUM	0	31-May-09	NDA	A/Wisconsin/67/2005
ITSDG-001	106016	DPD	SERUM	0	31-May-09	<20	A/Wisconsin/67/2005
ITSDG-001	106143	MRW	SERUM	0	31-May-09	NDA	A/Wisconsin/67/2005
ITSDG-001	105821	S-M	SERUM	0	31-May-09	NDA	A/Wisconsin/67/2005
ITSDG-001	106173	EVS	SERUM	0	31-May-09	NDA	A/Wisconsin/67/2005
ITSDG-001	105917	NCH	SERUM	-2	29-May-09	80	A/Wisconsin/67/2005
ITSDG-001	106100	LJF	SERUM	-2	29-May-09	NDA	A/Wisconsin/67/2005
ITSDG-001	106031	JGT	SERUM	-2	29-May-09	NDA	A/Wisconsin/67/2005
ITSDG-001	105912	JDF	SERUM	-2	29-May-09	NDA	A/Wisconsin/67/2005
ITSDG-001	105725	ELS	SERUM	-2	29-May-09	NDA	A/Wisconsin/67/2005
ITSDG-001	103852	MMI	SERUM	-2	29-May-09	NDA	A/Wisconsin/67/2005
ITSDG-001	100888	JTM	SERUM	-2	29-May-09	320	A/Wisconsin/67/2005
ITSDG-001	100973	G-B	SERUM	-2	29-May-09	NDA	A/Wisconsin/67/2005
ITSDG-001	105807	R-B	SERUM	-2	29-May-09	20	A/Wisconsin/67/2005
ITSDG-001	102985	N-W	SERUM	-2	29-May-09	NDA	A/Wisconsin/67/2005
ITSDG-001	D01	JGT	SERUM	28	26-Jun-09	80	A/Wisconsin/67/2005
ITSDG-001	D02	LJF	SERUM	28	01-Jul-09	40	A/Wisconsin/67/2005
ITSDG-001	D03	NCH	SERUM	28	01-Jul-09	160	A/Wisconsin/67/2005
ITSDG-001	D04	JTM	SERUM	28	30-Jun-09	320	A/Wisconsin/67/2005
ITSDG-001	D05	G-B	SERUM	28	01-Jul-09	160	A/Wisconsin/67/2005
ITSDG-001	D06	ELS	SERUM	28	01-Jul-09	NDA	A/Wisconsin/67/2005
ITSDG-001	D07	MMI	SERUM	28	29-Jun-09	NDA	A/Wisconsin/67/2005
ITSDG-001	D08	N-W	SERUM	28	30-Jun-09	NDA	A/Wisconsin/67/2005
ITSDG-001	D09	R-B	SERUM	28	29-Jun-09	320	A/Wisconsin/67/2005
ITSDG-001	R01	ESK	SERUM	28	29-Jun-09	NDA	A/Wisconsin/67/2005
ITSDG-001	R02	R-R	SERUM	28	29-Jun-09	NDA	A/Wisconsin/67/2005
ITSDG-001	R03	S-M	SERUM	28	01-Jul-09	NDA	A/Wisconsin/67/2005
ITSDG-001	R04	RJG	SERUM	28	01-Jul-09	NDA	A/Wisconsin/67/2005
ITSDG-001	R05	FLA	SERUM	28	01-Jul-09	NDA	A/Wisconsin/67/2005
ITSDG-001	R06	MRW	SERUM	28	30-Jun-09	NDA	A/Wisconsin/67/2005
ITSDG-001	R07	G-T	SERUM	28	30-Jun-09	NDA	A/Wisconsin/67/2005
ITSDG-001	R08	H-J	SERUM	28	29-Jun-09	NDA	A/Wisconsin/67/2005
ITSDG-001	R09	MTC	SERUM	28	30-Jun-09	NDA	A/Wisconsin/67/2005
ITSDG-001	R10	TSG	SERUM	28	30-Jun-09	NDA	A/Wisconsin/67/2005
ITSDG-001	R11	MPR	SERUM	28	30-Jun-09	NDA	A/Wisconsin/67/2005
ITSDG-001	R12	JDF	SERUM	28	29-Jun-09	28	A/Wisconsin/67/2005
ITSDG-001	R13	DRT	SERUM	28	30-Jun-09	NDA	A/Wisconsin/67/2005
ITSDG-001	R14	DPD	SERUM	28	29-Jun-09	NDA	A/Wisconsin/67/2005
ITSDG-001	R15	EVS	SERUM	28	01-Jul-09	NDA	A/Wisconsin/67/2005

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