SURVIVAL AND TRANSMISSION OF CORONAVIRUSES IN THE HEALTHCARE ENVIRONMENT

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Environmental Sciences and Engineering, School of Public Health.

Chapel Hill
2008

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ABSTRACT

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Survival and Transmission of Coronaviruses in the Healthcare Environment
(Under the direction of Dr. Mark Sobsey)

The need for a comprehensive understanding of the routes by which viruses can spread in healthcare environments and the measures needed to prevent transmission has taken on particular urgency since the advent of severe acute respiratory syndrome (SARS). First emerging in 2003, this newly discovered coronavirus infection spread through 26 countries, with over 8000 cases and 700 deaths. One of the striking features of the SARS outbreak was its spread in healthcare facilities, resulting in transmission to patients, visitors, and healthcare workers (HCWs). Evidence suggests that in addition to droplets and aerosols, environmental surfaces, including protective equipment worn by healthcare workers, may serve as vehicles for transmission of SARS-CoV in the healthcare environment. However, there are significant gaps in our knowledge of how coronaviruses survive on inanimate surfaces and objects, including personal protective equipment (PPE) items, found in healthcare environments. To fill these crucial knowledge gaps, this research was undertaken to better understand risks of viral contamination during PPE removal and the effects of temperature and humidity on the survival of coronaviruses on surfaces found in healthcare environments. These studies showed that currently recommended methods for removal of healthcare PPE are insufficient to protect HCWs from viral contamination during PPE removal, and that potential alternative methods for PPE removal should be developed and validated. Viral survival studies using human and

animal coronaviruses as potential surrogates for SARS coronavirus show that if deposited in high numbers, coronaviruses dried onto surfaces may survive for days at temperatures and humidity levels found in healthcare environments. These viruses may also survive on materials used to make PPE long enough to pose a transmission risk. These findings suggest other members of the coronavirus family could serve as conservative surrogates for modeling the risk of indirect personal contact and environmental transmission of SARS by healthcare surfaces and PPE items, and can be used in studies to determine ways to interrupt this route of exposure and reduce the risk of disease transmission.

To Kevin

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Mark Sobsey, and my committee, David Weber, Bill Rutala, Fred Pfaender, and Jan Vinjé, for their guidance. I would also like to thank Drs. Ralph Baric, Patrick Harrington, and Dean Erdman for technical advice and their generous sharing of coronavirus strains, Maria Gergen for technical assistance, Edie Alfano-Sobsey for assistance with fit-testing for the PPE work, Greg Samsa for statistical assistance, and Douglas Wait and Dorothy Thompson of the Sobsey lab for all that they have done. A special thanks to the Sobsey lab group for the friendship and support they have given me on a long road. This research was supported by the Centers for Disease Control and Prevention and was also supported in part by a grant (RR00046) from the General Clinical Research Centers program of the Division of Research Resources, National Institutes of Health.

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CHAPTER 1

Introduction and Background

Viruses have long been recognized as a source of nosocomial infections, including coronaviruses, influenza, mumps, measles, respiratory syncytial virus, varicella, rubella, adenoviruses, and noroviruses, with both humans and the physical environment in healthcare facilities playing a role in the spread of these viruses and the illnesses they cause (Sepkowitz, 1996; Sattar, 2004). The need for a comprehensive understanding of the routes by which viruses can spread in healthcare environments and the measures needed to prevent transmission has taken on particular urgency since the advent of severe acute respiratory syndrome (SARS). First emerging in 2003, the outbreak of this previously unknown atypical viral pneumonia become emblematic of infectious disease in the age of global travel, spreading from person to person, through cities, between countries, and across continents with the movements of human beings. When it was realized that an outbreak had begun, the race was on to find the etiologic agent. The finish line was first crossed by two groups almost simultaneously, identifying the causative agent of SARS as a novel member of the coronavirus family (Drosten *et al.*, 2003; Ksiazek *et al*., 2003 Rota *et al.* 2003). The virus was not only unknown up to that point, but unrelated to any of the currently known human or animal coronaviruses. This new virus, SARS coronavirus (SARS-CoV), spread through 26 countries, with over 8000 cases and 700 deaths before the chain of natural transmission was broken in late 2003 (WHO, 2004).

One of the striking features of the SARS outbreak was its spread in healthcare facilities, resulting in transmission to patients, visitors, and healthcare workers (HCWs) (McDonald *et al.*, 2004). The impact on HCWs in outbreak settings was significant; physicians (Chen *et al.*, 2004), medical students (Wong *et al.*, 2004), nurses (Loeb *et al.*, 2004), and emergency room personnel (Chen *et al.*, 2004) became infected with SARS in the course of patient care, accounting for approximately 20% of cases by the time the outbreak was contained (Chan-Yeung, 2004; Lau *et al.*, 2004).

The spread of SARS in healthcare facilities focused attention on the role of surfaces and other fomites in spreading nosocomial viral infection. SARS-CoV nucleic acids have been identified on surfaces in hospitals where SARS outbreaks took place, illustrating that SARS-CoV may be deposited on hospital surfaces, which may then serve as a reservoir for subsequent transmission (Chen *et al.*, 2004; Dowell *et al.*, 2004, Booth *et al.*, 2005). These studies only examined nucleic acids, and could not make any conclusions about the presence of infectious SARS-CoV. However, previous research has shown that other enveloped viruses can survive on surfaces. Non-SARS coronaviruses, which have been previously recognized as a source of nosocomial infections (Gagneur *et al.*, 2002), can survive on surfaces for up to 6 hours (Sizun, Yu, and Talbot, 2000). Results of laboratory experiments with SARS on surfaces have found that SARS-CoV survives up to 9 days in the presence of 10% FCS and up to 6 days without FCS (Rabenau *et al.*, 2005).

The possible survival of SARS-CoV on surfaces and objects has important infection control implications. Viral contamination of inanimate objects has been suggested as the vehicle for outbreaks of nosocomial viral infection (Roger *et al.*, 2000). Controlled

studies have shown that when people come in contact with inanimate surfaces, they can both deposit viruses on these surfaces and acquire viruses from them (Rheinbaben *et al.*, 2000; Rusin, Gerba, and Maxwell, 2002), and viruses on hands can be transferred to the face during hand-to-face contact (Rusin, Gerba, and Maxwell, 2002). Once deposited on surfaces, some pathogenic viruses can remain viable for hours to days, even on porous materials such as gowns and lab coats (Brady, Evans, and Cuartas, 1990; Bean *et al.*, 1982; Mbithi, Springthorpe, and Sattar, 1991; Sattar, Lloyd-Evans and Springthorpe, 1986). Given that surfaces are a possible source for acquisition of SARS-CoV in the healthcare environment, and that the few available studies suggest possible long-term survival, more data are needed on the survival characteristics of this virus in healthcare environments, and the effect of environmental variables such as temperature and humidity on viral survival rates. Generating such data is complicated by the fact that SARS-CoV causes a possibly fatal droplet- and aerosol-transmitted disease, and can be handled only by trained personnel under biosafety level 3 conditions, restricting it to a few specialized laboratories. Even restricted to high containment laboratories, such research carries risks There have been cases of laboratory-acquired SARS that occurred after the chain of natural transmission had been broken (Lim *et al.*, 2004), posing a risk of reintroducing SARS into human populations. For a pathogen with these characteristics, the use of a surrogate virus for studying patterns of survival and transmission on surfaces is desirable. Studies using other members of the *Coronaviridae* may be able to provide insight into the survival, persistence, and transmission risks of SARS-CoV and other nosocomial coronaviruses on surfaces in the healthcare environment.

Surfaces and fomites may not be the only vehicles of nosocomial coronavirus transmission. An outbreak of SARS in a large apartment complex in Hong Kong suggested a role for contaminated water droplets and aerosols in the transmission of SARS. It was found that SARS-CoV shed in the feces of an infected individual visiting an apartment in one of the buildings of the complex may have spread via viral aerosols that entered the bathrooms of other apartments through faulty toilet plumbing and floor drains, transmitting SARS to other occupants of the building (McKinney *et al.*, 2006). During outbreaks, it is possible that water becomes contaminated with SARS-CoV shed by infected individuals and that this water is subsequently aerosolized to serve as a vehicle of transmission.

In addition, sinks, water baths, and whirlpools have been identified as foci for the spread of nosocomial infections (Squier, Yu, and Stout, 2000). These locations are likely to have standing water for extended periods. If they become contaminated with SARS-CoV from infected patients or the hands of healthcare workers caring for infected patients, virus may survive and remain infectious in water (Wang *et al.*, 2005). Data on the survival of SARS in both contaminated and potable waters can help in quantifying and assessing the risk involved in this potential route of nosocomial spread.

Although contaminated water droplets and aerosols are thought to have played a part in the transmission of community-acquired SARS, droplets and aerosols from the respiratory secretions of infected patients were a more common route of spread in healthcare environments. Several studies were done to determine how to protect HCWs from such transmission. Epidemiologic studies of the spread of SARS in healthcare environments established a crucial role for personal protective equipment (PPE),

including gowns, masks, and gloves, in preventing the spread of SARS to healthcare workers (Chen *et al.*, 2004; Dwosh *et al.*, 2003; Lau *et al.*, 2004; Loeb *et al.*, 2004; Seto *et al.*, 2003). Although PPE certainly plays an important role in protecting healthcare workers from SARS and other more common respiratory pathogens (Gamage *et al.*, 2002), PPE is itself a kind of surface, and viruses can survive on the types of materials PPE is made from (Bean *et al.*, 1982; Brady, Evans, and Cuartas 1990; Lai, Cheng, and Lim, 2005). Thus, items of PPE themselves may play a role in the transmission of disease if they become contaminated with infectious viruses. This exposure route has been recognized by the Centers for Disease Control and Prevention (CDC), which has a protocol outlining the proper sequence of removal of PPE items to minimize the risk of contamination to the wearer during removal (CDC, 2005). However, there is no empirical evidence proving that this protocol does or does not prevent the spread of viruses from contaminated PPE to the wearer during removal. The risk of viral transmission from contaminated PPE is also difficult to assess because data are lacking on how coronaviruses and other nosocomial viruses survive on the materials used to make PPE. Therefore, the possibility that PPE itself may be an environmental surface that contributes to the spread of viruses such as SARS-CoV remains research question in need of being addressed. Like the role of healthcare surfaces and reservoirs for coronavirus, this is another research question that might be addressed with the use of surrogate viruses to model survival and transmission dynamics.

In summary, evidence from laboratory studies, environmental surveys, and epidemiologic studies suggests that environmental surfaces, including protective equipment worn by healthcare workers, may serve as vehicles for transmission of SARS-

CoV in the healthcare environment. However, there are significant gaps in our knowledge both of how viruses may spread to healthcare workers in the course of using PPE, and how coronaviruses themselves survive on inanimate surfaces and objects, including PPE items, found in healthcare environments. To fill these crucial knowledge gaps, this research was undertaken with the following objectives:

Research Objectives

- Using MS2 as a model, develop methods for the recovery of infectious virus from multiple types of healthcare personal protective equipment, including gowns, masks, gloves, and goggles.
- Using these virus recovery methods, estimate the probability that healthcare workers wearing virus-contaminated PPE who remove it using a US CDC protocol designed to minimize wearer contamination during removal will transfer virus to their hands, face, clothing worn underneath PPE, and to uncontaminated PPE.
- Determine the survival rates of infectious coronaviruses in clean water and water contaminated with human fecal matter, using transmissible gastroenteritis virus and mouse hepatitis virus as surrogates.
- Determine the effect of temperature and humidity on the survival rates of two human and two animal coronaviruses on hard, nonporous (stainless steel) surfaces.
- Determine the effect of porous and nonporous surfaces found in healthcare environments, including plastic, ceramic, laminate, fabrics, and glove materials, on the survival rates of coronaviruses.

Review of the Literature

SARS coronavirus

Severe Acute Respiratory Syndrome (SARS) was first recognized in February 2003 as an atypical pneumonia in an outbreak in Guandong, China (Rosling and Rosling, 2003). The first alert on SARS came from the World Health Organization in March 2003, as the illness spread to Vietnam, Hong Kong, Canada, Taiwan, Thailand, and Singapore (Parry, 2003). By May, the etiologic agent was identified almost simultaneously by two groups as a novel coronavirus (Rota *et al.* 2003; Drosten *et al.*, 2003). The virus was not only unknown up to that point, but also unrelated to any of the currently known human or animal coronaviruses. This new virus, SARS coronavirus (SARS-CoV), spread through 26 countries, with over 8000 cases and 700 deaths before the chain of natural transmission was broken in late 2003 (WHO, 2004).

The *Coronaviridae*, members of the order Nidovirales, are enveloped, single-stranded positive-sense RNA viruses approximately 60-200 nm in size. The family is divided into three groups, with Groups 1 and 2 containing the mammalian coronaviruses. They have an internal helical RNA-protein nucleocapsid, and an RNA genome that is `27–32 kb, with a 5' methylated cap and 3' poly-A. The structural proteins are the nucleocapsid (N) protein, complexed with genome RNA; the transmembrane glycoprotein (M); the spike protein (S); and the membrane associated E protein. Some Group 2 viruses contain an additional structural protein, the hemagglutinin-esterase protein (HE) (Navas-Martin and Weiss, 2003). Although analysis of the genome of SARS coronavirus suggests it is a possible late offshoot of the Group 2 coronaviruses (Snijder *et al.*, 2003), sequence comparison does not place it in any of the currently described groups of coronaviruses

(Marra *et al.*, 2003; Ruan *et al.*, 2003), suggesting that it belongs in its own group within the *Coronaviridae* family. The polymerase gene sequence of SARS-CoV has similarities to the Group 2 coronaviruses, but this virus is not a host-range mutant of a known coronavirus, and is not a result of recombination between other known coronaviruses (Holmes and Enjuanes, 2003).

SARS infection

Acquisition

SARS is transmitted by droplets (Seto *et al.*, 2004) and aerosols (Li *et al.*, 2005); protection against droplet exposures has been shown to lower the risk of infection (Seto *et al.*, 2004). Further evidence for respiratory acquisition came from several studies demonstrating that wearing masks was protective against infection (Dwosh *et al.*, 2003; Jefferson *et al.*, 2008; Loeb *et al.*, 2004; Seto *et al.*, 2003). Virus could be transmitted both by close contact with infected patients (Wong *et al.*, 2004), and could travel fairly long distances (i.e., multiple building floors) on air currents, as seen in a large apartment complex outbreak (Yu *et al.*, 2004). The basic reproduction number for SARS (the average number of secondary cases generated by one primary case in a susceptible population) was initially estimated at 2.7 in Hong Kong, until reductions in onset-to-hospitalization time, population contact rate, and hospital transmission reduced the reproduction number to drop to 1.0 (Riley *et al.*, 2003). This suggests that during an outbreak, before transmission is recognized and control measures are put in place, one infected individual could generate two more infections. The infectious dose of SARS-CoV is unknown. Using animal models, 10^6 median tissue culture infectious dose units ($TCID_{50}$) have been shown to induce experimental infection in cats and ferrets (Martina

et al., 2003), and experiments using 10^3 or $10TCID_{50}$ have induced viral replication in the lungs of ferrets (ter Meulen *et al*., 2004), but the dose-response relationship is not known.

Clinical course

The WHO case definition of SARS was fever of 38°C or more, cough or shortness of breath, new pulmonary infiltrates on chest radiography, and a history of exposure to a patient with SARS or absence of response to empirical antimicrobial coverage for typical and atypical pneumonia (β lactams and macrolides, fluoroquinolones, or tetracyclines). SARS infection is characterized by fever on presentation, chest x-ray abnormalities, chills and/or rigor, myalgia, cough, and headache. SARS is an infection with both respiratory and enteric involvement, as seen in two Hong Kong outbreaks. In one, diarrhea was seen in 73% of patients (Peiris *et al*., 2003). In another, 38% of 140 patients had watery diarrhea, without the presence of blood or mucus. More significantly, evidence of viral replication was found in biopsied intestinal tissues (Leung *et al*., 2003), indicating that the intestine is a site of active viral replication. However, the most severe effects of the disease are a result of respiratory compromise. The course of illness as charted by Peiris *et al*. (2003) for 75 patients in Hong Kong showed that 20% developed adult respiratory distress syndrome and required mechanical ventilation, and 7% died, of myocardial infarction, sepsis, and ARDS. Peires *et al*. described the clinical course:

> Week 1: fever, myalgia, and other systemic symptoms that generally improve after a few days. The increasing viral load during this phase suggests that the symptoms are largely related to the effect of viral replication and cytolysis.
>
> Week 2: the patients frequently had recurrence of fever, onset of diarrhea, and oxygen desaturation. Strikingly, nearly half the patients had shifting radiographic shadows. If viral-induced damage was the primary pathological mechanism, such a flitting pattern of radiological change is difficult to explain. The timing of the IgG seroconversion, which starts on day 10, seems to correlate with falls in viral load, which occurs from between day 10 and 15, despite the use of pulse methylprednisolone. Severe clinical worsening also occurs at this time, which cannot be explained by uncontrolled viral replication. This finding is supported by the progressive decrease in rates of viral shedding from nasopharynx, stool, and urine

from day 10 to 21 after onset of symptoms in the 20 patients who underwent prospective follow-up with RT-PCR.

20% of patients in this cohort progressed to the third phase, characterized by ARDS necessitating ventilatory support. Inevitably, several patients developed nosocomial sepsis during this phase of end-organ damage and severe lymphopenia.

Virus shedding and viral load

RT-PCR analysis of samples from this patient series found SARS-CoV RNA in nasopharyngeal aspirates, urine, and stool. Viral load in nasopharyngeal aspirates peaked on day 10 of infection, at 7.3 $\log_{10}$ RNA copies/mL (Peires *et al*., 2003). Chu *et al*., (2004) found that viral load in nasopharyngeal specimens collected at patient presentation were as high as 8.8 $\log_{10}$ RNA copies/mL, and that viral load was associated with eventual mortality. A study of 150 Hong Kong patients found the highest viral load by RT-PCR was highest for stool specimens with 6.1 $\log_{10}$ copies/mL, followed by urine with 4.4 $\log_{10}$ copies/mL and nasopharyngeal specimens with 2.4 $\log_{10}$ copies/mL. Again, viral load in nasopharyngeal aspirates was associated with mortality (Hung *et al*, 2004). Peak viral load in throat washes from patients in a Taiwan outbreak ranged from 3-4 $\log_{10}$ RNA copies/mL (Wang *et al*., 2004). Although PCR titer likely overestimate the load of infectious virus in patient secretions, the viral loads in these studies suggest that levels of infectious virus in body fluids may be high enough to pose a risk of infection to HCWs who come in contact with patient secretions, but more information about the dose-response relationship of SARS-CoV is needed.

SARS in healthcare workers

Droplets and aerosols were identified as the principal transmission routes of SARS (Li *et al.*, 2003; Tang *et al.*, 2006). One of the striking features of the SARS outbreak was its spread in healthcare facilities, resulting in transmission to patients, visitors, and HCWs.

There were multiple instances of SARS in healthcare facilities in several countries. A summary of these outbreaks is shown in Table 1-1.

Table 1-1. Outbreaks of SARS in healthcare facilities

Site	Facility type	Outbreak	Reference
Singapore	Tertiary acute care hospital	Single index patient led to five clusters of infection; 24 healthcare workers, 15 patients, 12 visitors affected	Chow *et al.*, 2004
Toronto	Community hospital	Began with relative of Toronto index case; eight nurses affected	Loeb *et al.*, 2004
Toronto	hospital	Case in nurse linked to participation in resuscitation of SARS patient	Christian *et al.*, 2004
Toronto	hospital	Began with relative of Toronto index case; 128 SARS cases (72 probable, 56 suspect)	Varia *et al.*, 2003
Toronto	hospital	Second wave of SARS transmission; linked to continued unrecognized SARS transmission in a community hospital after infection control measures were relaxed	CDC, 2003
Taiwan	municipal hospital	exposure from a "super-spreader" with SARS and an infected hospital laundry attendant who continued working while ill; at least 137 probable cases, including 45 in healthcare workers.	McDonald *et al.*, 2004
Taiwan	University hospital	index case came through ER, linked to municipal hospital outbreak; three clusters of SARS in patients, family members, and HCWs	Chen *et al.*, 2004
Hong Kong	Five hospitals	77 probable and suspected cases	Lau *et al.*, 2004
Hong Kong	community hospital	40 hospital workers infected; included physicians, nurses, health assistants	Ho *et al.*, 2003
Ontario	community hospital	10 suspected cases among hospital staff	Dwosh *et al.*, 2003
Hong Kong	teaching hospital	cluster of infections among medical students exposed to a SARS case	Wong *et al.*, 2004

The impact of SARS on HCWs in outbreak settings was highly important. Physicians, medical students, nurses, and support personnel became infected with SARS in the course of patient care, accounting for approximately 20% of known total cases by the time the outbreak was contained (Chan-Yeung, 2004; Lau *et al.*, 2004). This awareness of infection risk led to several investigations of the role of personal protective equipment for protecting HCWs from SARS, including epidemiologic studies examining the role of PPE in protecting HCWs in facilities treating SARS patients.

Personal protective equipment (PPE) is a vital tool in healthcare environments to protect healthcare workers (HCWs) from exposure to human pathogens during patient care activities. PPE refers to a variety of barriers and respirators used alone or in combination to protect mucous membranes, airways, skin, and clothing from contact with infectious agents. It includes liquid-proof gowns, face masks, gloves, and eye shields, which prevent occupational exposure to pathogens causing tuberculosis, influenza, measles, varicella, and other infectious diseases (Siegel *et al.*, 2007). Studies demonstrated that use of PPE could protect HCWs from SARS infection in the workplace. In a Hong Kong study of HCWs exposed to SARS patients, not wearing masks was significantly associated with illness (Seto *et al.*, 2003). A case-control study in Toronto found inconsistent use of PPE was associated with higher risk of infection (Lau *et al.*, 2004). A retrospective cohort study of nurses at a Toronto hospital found use of masks was associated with a significantly lower risk of infection (Loeb *et al.*, 2004). Another study showed that cases of SARS among hospital staff all occurred before respiratory precautions, including wearing of PPE, were begun. After all staff were required to wear PPE for respiratory protection, no additional staff members became infected (Dwosh *et*

al., 2003). Meta-analysis of published studies found that masks, (OR=0.32, 95% CI 0.25 to 0.40), N95 respirators (OR=0.09, 9% CI 0.03 to 0.30) and gloves (OR=0.43, 95% CI 0.29 to 0.65) were effective in preventing the transmission of SARS. Handwashing, masks, gloves, and gowns combined were also found to be protective (OR=0.09, 95% CI 0.02 to 0.35) (Jefferson *et al.*, 2008). Epidemiologic studies of PPE in preventing SARS transmission are summarized in Table 1-2.

Table 1-2. Epidemiologic studies of PPE and SARS infection among healthcare workers

Study type	Population	Findings	Reference
Matched case-control	HCWs in a cluster of 5 Hong Kong hospitals	Inconsistent use of PPE associated with SARS infection; possible PPE shortages	Lau *et al.*, 2004
Case-control	Doctors and nurses in a hospital in Hanoi, Vietnam	Use of masks was significantly protective against SARS transmission	Nishiura *et al.*, 2005
Case-control	HCWs in a cluster of 5 Hong Kong hospitals	mask, gloves, gowns, and hand-washing evaluated; all infected staff had omitted at least one measure; masks significantly protective against infection	Seto *et al.*, 2003
Case-control	HCWs in a Singapore hospital	N95 masks were significantly protective against SARS	Teleman *et al.*, 2004
Matched case-control	Probable SARS cases in Beijing	Masks were strongly protective against SARS	Wu *et al.*, 2004
Case- Control	staff members from ten hospitals in Guangdong, China	Masks and goggles were significantly protective	Yin *et al.*, 2004; Jefferson *et al.*, 2008
Retrospective cohort	Nurses in two Toronto critical care units	N95 masks were significantly protective against SARS	Loeb *et al.*, 2004

Although PPE plays an important role in preventing transmission of infectious diseases from patients to healthcare workers, there were concerns that contaminated PPE

may play a role in the spread of infectious agents when it is handled during removal. To minimize the risk of HCW contamination during the removal of personal protective equipment, the Centers for Disease Control and Prevention designed a protocol for the safe removal of PPE (Siegel *et al*, 2007). However, the risks of pathogen exposure from contaminated PPE are not well understood. It is possible that items of PPE that become contaminated could serve as fomites, transferring infectious organisms from surfaces to hands and other surfaces. The frequency and efficiency of such transfer, however, is not known. Pittet *et al* (2006) described five steps necessary for pathogens to be transferred from patient to patient via healthcare workers' hands (Figure 1-1).

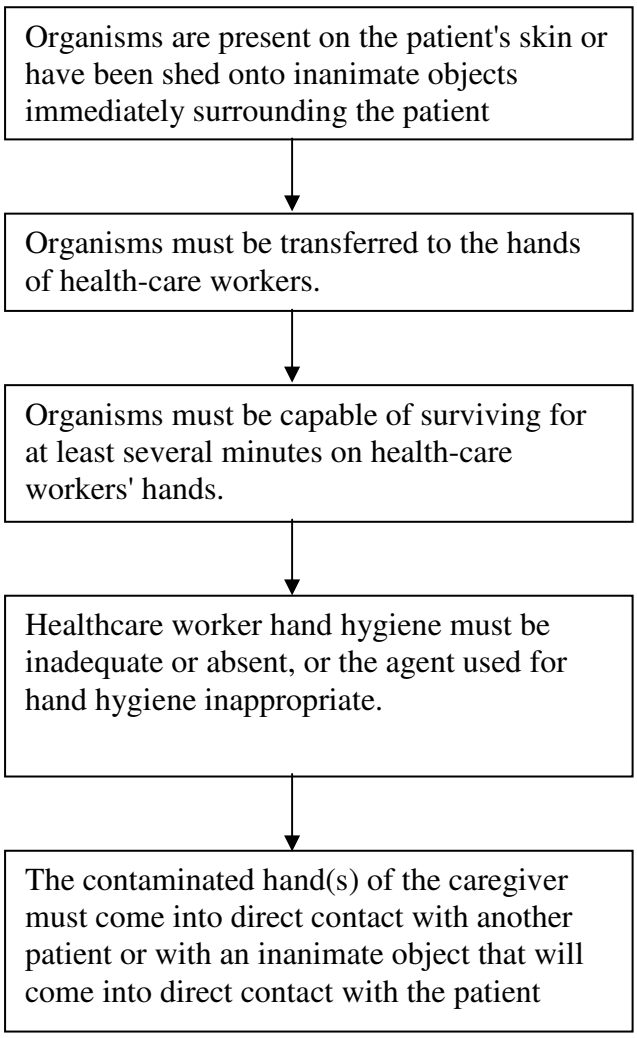

Figure 1-1. Sequence of events for transfer of organisms via hands in healthcare environments (from Pittet *et al.*, 2006).

Contaminated PPE can play a role in steps 1-2. If patients shed viruses onto healthcare workers' PPE in the course of patient care, these viruses can remain infectious when PPE is removed, and subsequent transfer of viruses from contaminated PPE to hands is a possibility. Transfer of viruses between hands and experimentally contaminated inanimate objects has been demonstrated in controlled laboratory experiments. Materials tested include stainless steel, fabrics (Rusin, Maxwell, and Gerba, 2002), plastic (Gwaltney and Hendley, 1982), and gloves (Hall, Douglas, and Geiman, 1980). Studies

of viral transfer between hands and fomites demonstrate variable efficiency of transfer depending on surface type and virus type, with transfer efficiency reduced substantially by drying of the virus on the surface. These studies are summarized in Table 1-3.

Table 1-3. Studies of virus transfer between hands and surfaces

Transfer type	Virus	Efficiency	Reference
Hand to stainless steel; stainless steel to hand	rotavirus	16% 20 min after hand contamination; 2% 60 min after hand contamination	Ansari *et al.*, 1998
Hand to stainless steel; stainless steel to hand	hepatitis A	disk to hand: 28% 20 min after hand contamination; 10% 60 min after hand contamination Hand to disk: 36% 20 min after hand contamination; 26% 60 min after hand contamination	Mbithi *et al.*, 1992
stainless steel to hands	rhinovirus	0.58% after drying of virus	Sattar *et al.*, 1993
Hand to stainless steel; stainless steel to hand	rhinovirus	hand to disk, 0.92% after 20 min drying; disk to hand, 0.67% after 20 min drying	Ansari *et al.*, 1991
Hand to stainless steel; stainless steel to hand	human parainfluenza	hand to disk, 0% after 20 min drying; disk to hand, 1.48% after 20 min drying	Ansari *et al.*, 1991
metal faucet to hand	bacteriophage PRD-1	33% transfer	Rusin, Maxwell, and Gerba, 2002
fabric to hand	bacteriophage PRD-1	<0.01% transfer	Rusin, Maxwell, and Gerba, 2002
plastic to hand	rhinovirus	5/10 recipients infected after exposure to contaminated cup handles; 9/16 infected after exposure to contaminated plastic tiles.	Gwaltney and Hendley, 1982
latex glove to hand	respiratory syncytial virus	transfer from latex gloves to fingers up to 25 min after contamination	Hall, Douglas, and Geiman, 1980

These studies of experimental transfer suggest the possibility that viruses can transfer from PPE to hands when contaminated items are handled in the course of PPE removal and disposal. In addition, contamination can be present on skin after exposure to pathogens even when PPE is worn (Zamora *et al.*, 2006), and may be transferred to used items of PPE if they are handled after removal. Virus transfer between hands and PPE items can encourage both accidental autoinoculation by the healthcare worker and subsequent transmission of viruses to other patients, staff, or family members, especially when inadequate hand hygiene is practiced (Pittet *et al.*, 2006).

As indicated in (Figure 1-1).

Figure 1-1, organisms must be shed onto inanimate objects in order to begin the chain of transmission via hands. However, these organisms must survive on inanimate objects, such as PPE, long enough to be transferred to hands. There have been few studies assessing the survival of pathogens on materials used to make personal protective equipment (Yassi *et al.*, 2005). Studies of pathogen survival on N95 respirators have evaluated bacteria, and indicate that bacterial spores can survive up to 12 days with only 1-2 $\log_{10}$ reduction in infectious titer (Wang, 1999); other types of bacteria can survive on masks for days (Rengasamy *et al.*, 2004). Some investigations have found that viruses can survive on materials used to make other types of PPE. When deposited in high numbers (~10^6 $TCID_{50}$), SARS-CoV has been found to survive on gowns for up to 2 days (Lai, Cheng, and Lim, 2005). Enveloped ssRNA viruses have been shown to survive on latex glove material; respiratory syncytial virus can survive on gloves for up to 1.5 hours (Hall, Douglas, and Geiman, 1980). Human coronavirus 229E can survive on gloves for up to 2 hours, although it loses up to 85% of its infectious titer (Sizun *et al.*, 2000), and

avian influenza virus can survive for up to 6 days without loss of infectious titer (Tiwari *et al.*, 2005). Non-enveloped ssRNA viruses such as human rotavirus and hepatitis A virus can survive for several days on latex under ambient conditions, with only ~1 $\log_{10}$ loss in infectious titer (Abad, Pinto, and Bosch, 1994). These studies suggest that viruses have the potential to survive on PPE materials for much longer than single-use PPE is usually worn, creating the potential for viral transfer when PPE is handled after wearing.

Nosocomial viruses on surfaces

PPE items are not the only fomites that may play a role in the transmission of viral infection in healthcare environments. Environmental surfaces in hospital environments have been recognized as sources of nosocomial infection, possibly serving as reservoirs for viral pathogens such as influenza, norovirus, rotavirus and SARS-CoV (Hota, 2004). Possible evidence of SARS-CoV has been found on hospital surfaces during a SARS outbreak. Sampling of surfaces in hospitals during SARS outbreaks, including surfaces in patient rooms and areas on floors used for isolation of SARS patients, found SARS-CoV nucleic acids on surfaces and inanimate objects in patient rooms, nurses' stations, and public areas of the hospital (Booth *et al.*, 2005; Dowell *et al*, 2004). Infectious virus was not cultured from any of the samples, so it is unclear whether the presence of viral nucleic acid indicates that infectious SARS-CoV is present on hospital surfaces in outbreak settings. The presence of nucleic acids, however, suggests the possibility that infectious virus was present on these surfaces for a period of time, and these surfaces could become sources of further viral transmission.

Assessing the risk posed by SARS-CoV on surfaces requires data on how long viruses survive on inanimate surfaces and the influence of temperature, humidity, and surface

type on viral survival. Several studies have been done on the survival of SARS-CoV on surfaces, including paper, cotton, and various nonporous surfaces. Studies of SARS-CoV survival on surfaces are shown in Table 1-4.

Table 1-4. Survival of SARS-CoV on surfaces

Surface type	Survival	Reference
paper	24 hr when starting titer is 10^6 $TCID_{50}$; 3 hr when 10^5; <5 min when 10^4 (kinetics not studied)	Lai, Cheng, and Lim, 2005
disposable gown	2d when starting titer is 10^6 $TCID_{50}$; 24 hr when 10^5; 1 min when 10^4 (kinetics not studied)	Lai, Cheng, and Lim, 2005
cotton gown	24 hr when starting titer is 10^6 $TCID_{50}$; 1 hr when 10^5; 5 min when 10^4 (kinetics not studied)	Lai, Cheng, and Lim, 2005
polystyrene petri dish	4 $\log_{10}$ reduction in infectious titer over 9 days	Rabenau et al., 2005
plaster	24 hr (initial titer 10_4 $TCID_{50}$)	WHO, 2003
plastic	36 hr (initial titer 10_4 $TCID_{50}$)	WHO, 2003
formica	36 hr (initial titer 10_4 $TCID_{50}$)	WHO, 2003
stainless steel	36 hr (initial titer 10_4 $TCID_{50}$)	WHO, 2003
wood	12 hr (initial titer 10_4 $TCID_{50}$)	WHO, 2003
cotton fabric	12 hr (initial titer 10_4 $TCID_{50}$)	WHO, 2003
slide	72 hr (initial titer 10_4 $TCID_{50}$)	WHO, 2003
paper	24 hr (initial titer 10_4 $TCID_{50}$)	WHO, 2003

When deposited in high numbers, SARS-CoV can remain infectious for days on surfaces. Survival is generally longer on nonporous surfaces than porous surfaces. The data in Table 4 was gathered on SARS-CoV survival at room temperature. However, temperature and humidity were not controlled variables in these studies, making it difficult to ascertain the effect of these variables on SARS-CoV stability in the

environment. There are limited data available on the survival of other, non-SARS coronaviruses on surfaces, and similar to the data for SARS-CoV, they often did not measure the effects of temperature and humidity variation on survival. Data on human coronavirus survival on surfaces are shown in Table 1-5.

Table 1-5. Survival of non-SARS human coronaviruses on surfaces (from Sizun, Yu, and Talbot, 2000)

Surface type	Virus type	Survival
aluminum	229E	loses 80% of infectivity in 3 hr; decline of 5 $\log_{10}$ infectivity by 6 hr
sponge	229E	loses 90% of infectivity in 6 hr; decline of 5 $\log_{10}$ infectivity by 12 hr
latex	229E	loses 80% of infectivity in 3 hr; decline of 5 $\log_{10}$ infectivity by 6 hr
aluminum	OC43	decline of 5 $\log_{10}$ infectivity by 3 hr
sponge	OC43	decline of 5 $\log_{10}$ infectivity by 1 hr
latex	OC43	decline of 5 $\log_{10}$ infectivity by 1 hr

The results of these studies on human coronaviruses 229E and OC43 suggest that they are inactivated much more quickly on both porous and nonporous surfaces than is SARS-CoV. This suggests that these viruses may be of limited utility in predicting the survival of SARS-CoV on environmental surfaces. Given the paucity of available information on coronavirus survival on environmental surfaces, comparison to the survival of other viruses may provide some insight into the survival of SARS-CoV on environmental surfaces and the effect of variables such as temperature, humidity, and surface type on survival.

Virus survival on environmental surfaces

Effect of viral type and structure on survival

Studies of viral survival on surfaces have shown that virus type and structure affects survival on surfaces. The presence or absence of a lipid envelope affects how long viruses can survive on surfaces, with non-enveloped viruses generally having longer survival than enveloped viruses. Non-enveloped viruses can survive on nonporous surfaces for weeks with very little decline in titer (Abad, Pinto, and Bosch, 1994; Abad *et al.*, 2001), whereas enveloped viruses such as avian influenza can exhibit a 1-2 $\log_{10}$ decline in infectious titer over 72 hours and >3 $\log_{10}$ decline in 6 days (Tiwari *et al.*, 2006). Human influenza is inactivated relatively quickly, showing a 3 $\log_{10}$ decline in infectious titer after 24 hours on stainless steel (Noyce, Michels, and Keevil, 2007). Other enveloped viruses are inactivated even more rapidly on surfaces; cytomegalovirus, another enveloped virus, survives for only 8 hours on glass (Faix, 1984), and human respiratory coronaviruses OC43 and 229E lose 100% of their infectivity on metal surfaces within 3 hours (Sizun, Yu, and Talbot, 2000). Cell-free preparations of human immunodeficiency virus suspended in serum appear somewhat more resistant on surfaces, losing 2 $\log_{10}$ infectious titer per week (van Bueren *et al.*, 1994). Although both enveloped and non-enveloped viruses can persist on surfaces for days if deposited in high enough numbers, the evidence suggests that the rates of inactivation on surfaces are much higher for viruses with lipid envelopes than for non-enveloped viruses.

Effect of genome structure on survival

Previous studies of viral disinfection offer some insight into the possible effects of genome structure on virus susceptibility to disinfectants, and may provide useful information on mechanisms might influence virus survival on environmental surfaces. It

has been posited that double stranded DNA genomes, such as adenovirus, are more resistant to damage by UV radiation, as suggested by experiments showing that adenoviruses have greater resistance to inactivation by UV irradiation of water than ssRNA viruses. If one strand of a dsDNA genome is damaged, the other strand can still serve as a template, allowing host cell enzymes to repair damage to the viral nucleic acid (Thurston-Enriquez *et al.*, 2003).

Although differences in nucleic acid structure may explain the differential susceptibility of viruses to disinfectant agents, investigations of viral survival suggest that it is not the most important factor affecting survival of viruses dried onto surfaces. Other structural componenets of viruses besides their nucleic acids may influence the persistence of virus infectivity in the environment. Studies of adenovirus, a dsDNA virus, have shown that it may not survive as long on surfaces as some ssRNA viruses (Abad, Pinto, and Bosch, 1994; Abad *et al.*, 2001). Adenoviruses exhibit greater inactivation during desiccation than ssRNA viruses such as HAV and HRV (Abad, Pinto, and Bosch, 1994). This may be due to the presence of penton fibers on the adenovirus viral capsid, which mediate attachment to the host cell and are important for infection (Zubieta *et al.*, 2004). Damage to these fibers caused by desiccation may play a role in the loss of infectivity of this virus when dried onto surfaces (Sattar, personal communication). This is reinforced by the observation that bacteriophage B40-8, a dsDNA tailed phage, survives significantly longer on surfaces than adenovirus, and has inactivation rates more similar to those seen for hepatitis A virus (Abad, Pinto, and Bosch, 1994). Therefore, although dsDNA genomes may have greater stability or ability to undergo repair, this

may not be the most important virus structural factor influencing viral survival on surfaces; the fate of outer structural components of the virion may play a greater role.

More information on the role of genome structure on virus survival may be gleaned from studies comparing the survival of ssRNA and dsRNA viruses on surfaces. Hepatitis A, a ssRNA virus, survives significantly longer on aluminum surfaces when compared to the dsRNA human rotavirus, but rotavirus survived significantly longer on a nonporous surface, paper. On latex, there was no difference in the survival of the two viruses. Although the relationship between genome structure and survival varies by surface type for ssRNA and dsRNA, these same studies showed that rotavirus survived significantly longer on all surface types than did adenovirus (Abad, Pinto, and Bosch, 1994). Like adenovirus, rotavirus has viral capsid proteins, some with associated carbohydrates, that protrude from the capsid surface, and as in the case of adenovirus, these protruding proteins may be sensitive to desiccation. These results suggest that the outer capsid structures may play a greater role in viral survival on surfaces than the structure of the viral genome.

Effects of temperature on survival

Survival of non-enveloped viruses such as hepatitis A, poliovirus and adenovirus is enhanced at low temperatures (Abad, Pinto, and Bosch, 1994; Abad *et al.*, 2001; Mbithi, Springthorpe, and Sattar, 1991). Survival of rotavirus was enhanced at low temperatures at medium and high RH; the rate of virus inactivation at the high RH level at 4°C was significantly lower than at 22°C. (Sattar *et al.*, 1986). Enveloped viruses follow similar patterns; coronaviruses are inactivated more slowly at lower temperatures (Tennant, Gaskell, and Gaskell, 1994). This greater survival at lower temperature is similar to results observed for virus survival in aerosols (Ijaz *et al.*, 1985) and water (Yates, Gerba,

and Kelley, 1985; Enriquez, Hurst, and Gerba, 1995). The effect of temperature on virus survival on surfaces appears to be similar to the effects of temperature in other environmental media such as air and water.

Effects of humidity on survival

Studies on the relationship between virus survival and relative humidity has not elucidated a clear and consistent relationship. Survival of some non-enveloped viruses is greater at high relative humidity (Abad, Pinto, and Bosch, 1994). Studies of hepatitis A have found that between 25 and 95% RH, the survival of virus on surface is inversely proportional to the RH level. These investigations also found that the relationship between HAV survival and RH at 20°C was the same as at 5°C, with higher relative humidity levels resulting in shorter survival times. Although the basic relationship remained the same, the effect of humidity on survival was "less pronounced" at lower temperatures compared to higher temperatures (Mbithi, Springthorpe, and Sattar, 1991). Opposite results were found for human rotavirus on stainless steel, plastic, and glass; rates of viral inactivation were higher at 50% RH than at 25%, and higher at 80% RH than at 50%. Rotavirus could still remain infectious at 25% and 50% RH for up to 12 days with little loss of infectivity (Sattar *et al.*, 1986). Human rhinovirus behaved similarly; survival on surfaces was 14 hours at high RH but only 2 hr at low RH (Sattar *et al.*, 1987). In studies of effects of relative humidity and temperature on the survival of human rotavirus as a thin layer of faeces on an impervious surface and on absorbent material, virus infectivity was very stable at low and high relative humidities but not in the medium range of relative humidity (Moe and Shirely, 1982). Rotavirus infectivity declines more rapidly under all humidities at 37° C than at 4°C or 20°C. (Moe and Shirley, 1982) For enveloped viruses in aerosols, previous studies have reported greater

virus survival in at lower relative humidity than higher relative humidity. Semliki Forest Virus in aged aerosols lost its infectivity more rapidly as the humidity was increased (Benbough, 1961). This longer survival of viruses in aerosols at lower relative humidity has also been reported for vaccinia, influenza and Venezuelan equine encephalomyelitis viruses (Harper, 1963) The effects of low (10%), intermediate (35%) and high (90%) RH were studied at 23°C and with four aerosolized viruses: Newcastle disease virus, infectious bovine rhinotracheitis virus, vesicular stomatitis virus, and bacteriophageT3. Virus loss on aerosol generation was consistently lower at 90% than at 10 or 35% RH. When stored at 23°C, Newcastle disease virus and vesicular stomatitis virus survived best at 10% RH, while infectious bovine rhinotracheitis virus and bacteriophage T3 survived best at 90% RH (Songer, 1967).

The available data suggest that although rates of inactivation of most viruses are slower at lower temperatures, the effects on RH on virus survival differ among viruses. High relative humidity appears to enhance the survival of some viruses, low relative humidity appears to enhance the survival of other viruses, and for some viruses survival is better at low and high RH levels than at 50% RH. Therefore, the relationship between humidity and virus inactivation is still not entirely clear, and may differ by virus type (Abad, Pinto, and Bosch, 1994; Mbithi, Springthorpe, and Sattar, 1991).

Effects of surface type and porosity on survival

Virus survival has been observed to be shorter on porous than nonporous surfaces for some viruses, but other viruses show no difference. Non-enveloped viruses can persist for weeks on latex and paper surfaces with 1-3 $\log_{10}$ decline in infectious titer, compared to ~1 $\log_{10}$ on aluminum surfaces (Abad, Pinto, and Bosch, 1994). Abad *et al.* (2001) found similar survival of non-enveloped viruses on china and paper. Enveloped avian influenza

viruses can remain stable on latex for up to 6 days, which is longer than their survival on steel surfaces, but lose >1 $\log_{10}$ infectious titer within 48 hours on fabric (Tiwari *et al.*, 2006). No difference was observed in the survival of human coronaviruses 229E and OC43 on aluminum vs. latex surfaces (Sizun, Yu, and Talbot, 2000). On metal surfaces, the metal type can affect survival; under identical conditions, human influenza loses 2 $\log_{10}$ infectious titer on stainless steel in 6 hours, but 4 $\log_{10}$ on copper surfaces (Noyce, Michels, and Keevil, 2007). Rotavirus survival on paper and cloth was found to be highly variable, with virus recovered from cloth for days, but inactivated on paper immediately after drying (Sattar *et al.*, 1986). Results suggest that the effect of porous vs. nonporous surfaces may differ by virus type. In comparing both within and across studies, the effect of differences in viral elution efficiency from different types of surfaces is difficult to evaluate. It may be that the efficiency of virus elution from porous surfaces, such as fabrics, is lower than that of nonporous surfaces. In comparisons within the same studies using the same elution methods, however, it appears that the major differences in virus survival are found between hard nonporous surfaces (steel, aluminum, china) and porous surfaces (paper, fabric), while the effects of soft nonporous surfaces such as latex are less clear. Studies of virus survival on surfaces indicate a few trends:

- Non-enveloped viruses survive longer with less decline in infectivity than enveloped viruses
- lower temperatures reduce rates of viral inactivation compared to higher temperatures
- virus survival tends to be greater at high RH levels compared to low RH levels

Although some studies found a protective effect of high relative humidity, the effect of relative humidity on virus survival is not clear, and how the humidity level interacts with temperature to affect viral survival on surfaces is also not completely clear from the

current literature. There does not appear to be a definitive relationship between genome structure and survival on surfaces, although this is difficult to elucidate because much of the available literature examines only single-stranded RNA viruses. It may be that the kinetics of viral survival on surfaces are modulated by the effects of temperature, humidity, desiccation, and other variables on the outer structures of virions. Enveloped viruses, and viruses with protein and carbohydrate structures protruding from the outer capsid, appear to be more sensitive to the effects of desiccation than viruses without these structures, suggesting that oxidation of lipid envelopes and damage to protruding capsid proteins that mediate infection may play an important role in viral inactivation on surfaces. These modulating effects may differ among virus types. In order to fully understand the relationships among temperature, humidity, virus type, surface type, and survival, these experiments need to be replicated with multiple virus types under similar exposure conditions.

CHAPTER 2

Experimental design and methods

Selection of surrogate viruses

The family *Coronaviridae* consists of three taxonomic groups, encompassing human, mammalian, and avian viruses. Sequencing and genetic analysis of the SARS-CoV genome currently place it as a possible late offshoot of the Group 2 coronaviruses (Snijder *et al.*, 2003), but not a member of any of the currently recognized groups (Marra *et al.*, 2003; Ruan *et al.*, 2003), making it difficult to identify a member of the *Coronaviridae* most closely related to SARS-CoV. Because of its uncertain place within the family, surrogate viruses for studying the environmental survival of coronaviruses were chosen from Groups 1 and 2, which contain the mammalian coronaviruses (Group 3 has only one member, the avian infectious bronchitis virus). This allows for the collection of data to address possible differences in survival and persistence between the two groups of mammalian coronaviruses, and will allow for later comparison to SARS-CoV itself if future genetic analysis pinpoints more precisely its place within the family. Candidate surrogates were therefore chosen from two groups, with a human and an animal virus selected from each group: transmissible gastroenteritis virus (TGEV) and human coronavirus 229E from Group 1, and mouse hepatitis virus (MHV) from Group 2.

Surrogates for modeling the transmission of viruses during removal of personal protective equipment (PPE) needed to be 1) appropriate surrogates for viruses known to cause nosocomial infection and 2) acceptable for use in experiments with human

volunteers. The coronaviruses described above for use in survival experiments are either human respiratory pathogens or animal pathogens whose potential risk for zoonotic transmission would be difficult to assess, making them unacceptable for use with human volunteers.

Testing of the effectiveness of handwashing agents and of PPE materials as barriers against viruses has been done using bacteriophages as surrogates for pathogenic human viruses (Lytle and Baker, 1999; Sickbert-Bennett *et al.*, 2005). Some nosocomial viral pathogens, including SARS-CoV and influenza, are enveloped, suggesting an enveloped bacteriophage might be a candidate surrogate for modeling the behavior of enveloped viruses in these experiments. There is one such phage, Φ6. *Pseudomonas* phage Φ6 is a member of the *Cystoviridae*, an enveloped, dsRNA phage whose host bacterium is *Pseudomonas syringae* (Laurinavičius *et al.*, 2004). However, there are difficulties involved in working with this bacteriophage due to the fact that its host bacterium is a regulated plant pathogen that requires U.S. Department of Agriculture permits for possession (USDA, 2008).

Genome structure is also an important factor in the selection of a surrogate. Many of the viruses that have been identified as important causes of nosocomial viral infection or have high potential for nosocomial transmission, such as SARS-CoV, influenza, parainfluenza, mumps, measles, respiratory syncytial virus, and noroviruses, are single stranded RNA viruses. The dsRNA genome of Φ6 differs from that of most hospital-acquired viruses; the only nosocomial viral pathogen of interest with a dsRNA genome is the rotavirus (ICTV, 2002). This difference in genome composition is also a factor with bacteriophage ΦX174, a non-enveloped ssDNA phage of the family *Microviridae* (ICTV,

2002) commonly used for barrier testing of PPE (Lytle and Baker, 1999). Previous studies on environmental survival of viruses on surfaces have shown differences in survival between DNA and RNA viruses (Abad, Pinto, and Bosch, 1994), suggesting that a single-stranded RNA virus may be a more appropriate surrogate for modeling the behavior of common nosocomial viruses, as well as SARS-CoV.

Bacteriophage MS2, a virus of the family *Leviviridae,* genus *Levivirus*, is a 24-26 nm, non-enveloped, ssRNA virus (ICTV, 2002). MS2 has been extensively used as a surrogate for studying the survival and persistence of pathogenic human ssRNA viruses (Bae and Schwab, 2007; Bourget *et al.*, 2007; Dawson *et al.*, 2005; Enriquez *et al.*, 2003; Finch and Fairbairn, 1991; Helmer and Finch, 1993). It has also previously been used for testing the efficacy of hand hygiene agents in human volunteer studies, and has an acceptable safety profile for studies using human volunteers (Sickbert-Bennett *et al.*, 2005). Because of its similarity in genome composition to many important nosocomial viral pathogens, as well as its safety profile for use with human volunteers, MS2 was chosen as a surrogate virus for modeling the transfer of virus on healthcare PPE in human volunteer experiments.

Preparation of coronavirus stocks

TGEV and MHV were kindly provided by R. Baric, University of North Carolina, Chapel Hill; 229E and OC43 were kindly provided by D. Erdman, Centers for Disease Control and Prevention, Atlanta, GA. Cell lines were obtained from the American Type Culture Collection (Manassass, VA). Cell lines used were: swine testicular cells (ST, ATCC #CRL-1746) for TGEV; delayed brain tumor cells (DBT) for MHV; and human lung fibroblast cells (MRC-5, ATCC# CCL-171) for 229E. Viral stocks were propagated

by infecting confluent layers of host cell cultures in flasks, harvesting cell lysates, clarifying by centrifugation (3000×g, 30 min, 4°C), and storing resulting supernatants as virus stock at -80°C. Viral titers were determined by quantal assays on confluent host cell layers in 24-well plates and virus concentration was expressed as the most probable number (MPN).

Coronavirus Infectivity assays

TGEV and MHV were propagated and assayed in host cells with maintenance medium consisting of Eagle's minimum essential medium (MEM), 7.5% sodium bicarbonate, HEPES buffer, 10% bovine serum replacement (Fetal Clone II, Hyclone, Logan, UT), 10% lactalbumin hydrolysate, non-essential amino acids, 1 mM sodium pyruvate and gentamicin (0.1 mg/mL)/kanamycin (0.05 mg/mL). Coronavirus 229E was propagated and assayed in host cells with maintenance medium consisting of Eagle's minimum essential medium (MEM), 10% newborn calf serum (Hyclone, Logan, UT), 7.5% sodium bicarbonate, HEPES buffer, glutamine, non-essential amino acids, 1 mM sodium pyruvate, gentamicin (0.1 mg/mL)/kanamycin (0.05 mg/mL) and nystatin (100 U/mL).

Coronavirus survival in water

Reagent-grade water was produced from laboratory tap water by a Dracor™ water purification system (Dracor, Durham, NC) which includes reverse osmosis and ultraviolet light treatment. Lake water came from University Lake, an impoundment that serves as the drinking water source for the town of Chapel Hill, and was obtained from the raw water inlet of the Orange Water and Sewer Authority (OWASA) drinking water treatment plant. Settled sewage was obtained from the OWASA waste water reclamation facility,

and was pasteurized in a waterbath at 70°C for 3 hours to inactivate other microorganisms that would interfere with cell culture infectivity assays of coronaviruses.

For each virus, 5 mL of clarified virus stock was spiked into duplicate 45 mL aliquots of test water. A positive control sample for measuring the initial virus concentration in water at time 0 was taken and assayed immediately after spiking. One aliquot of test water was held at room temperature (23-25°C), and one was held at refrigerator temperature (4°C). At each time point, samples were taken and assayed for virus infectivity on the appropriate cell line. Four replicate samples were assayed at each time point. Virus survival at each time point was expressed as $\log_{10} (N_t/N_0)$, where N_t is the virus concentration in MPN/mL at time *t*, and N_0 is the initial virus concentration in MPN/mL in the positive control sample at time 0.

Data analyses were carried out using Excel 2003 (Microsoft Corp.), and GraphPad Prism 5 (GraphPad, San Diego, CA). The parameter $\log_{10} (N_t/N_0)$ versus time was used to perform regression analysis for each virus and water type. Coefficients from regression analysis were used to predict times needed for 90, 99, 99.9, and 99.99% reduction of each virus at each temperature.

Recovery of MS2 from personal protective equipment

Eluent comparison

Virus stocks were diluted in 0.01M phosphate buffered saline to the desired concentration. Inocula for experiments were titered using the double agar layer (DAL) plaque assay on tryptic soy agar medium using the host bacterium *E. coli* ATCC #15597 (EPA, 2001b). Virus was applied to 4 cm^2 swatches of contact isolation gown fabric in a single volume of 10µL. Swatches were held at room temperature in a biological safety

cabinet for 15 minutes, then immersed 250 mL sterile eluent solution. Eluent and PPE were agitated on a high-speed shaking platform for 20 minutes. Swatches were removed, eluent was expressed into the container by wringing, and the swatch was discarded. Eluent was diluted in tryptic soy broth (TSB) (Becton Dickinson) and assayed for MS2 infectivity using DAL.

Elution of viruses from personal protective equipment

Virus stocks were diluted in 0.01M phosphate buffered saline to the desired concentration. For selected experiments, viruses diluted in PBS were monodispersed after dilution by sequential passage through hydrophilic polycarbonate filters with pore sizes of 0.2 μm (Isopore, Millipore, Billerica, MA) and 0.08 μm (Nuclepore track-etched membranes, Whatman, England) pre-rinsed with 0.01% Tween 80 and sterile distilled water. Inocula for experiments were titered by DAL. Virus was applied to the surface of PPE in a single volume of 10μL. PPE items were held at room temperature in a biological safety cabinet for 15 minutes, then immersed in 1-2 L eluent solution (depending on size of item). Eluent and PPE were agitated on a reciprocal shaking platform at 120 cycles/min for 20 minutes. PPE was removed and discarded, and eluent was assayed for infectious virus using a two-step enrichment procedure for MS2 (EPA, 2001a).

Two-step enrichment method for MS2 infectivity assay

Eluent samples were diluted as needed using additional sterile eluent. For enrichment, 4M MgCl2 (12.5 mL/L) and 10X tryptic soy broth (50 mL/L) were added to the final dilutions. Appropriate dilutions were split into 10 replicate volumes per dilution, and log phase bacterial host (0.5 mL per tube), was added to each replicate volume. Enrichment samples were incubated for 24 hours at 37°C. Host bacterium cultures were also incubated at 37°C to check for contamination. For spot plates, 20 mL log phase bacterial

host was added to 1L half-strength tryptic soy agar (30g tryptic soy broth, 7.5g bacto agar per liter) at 45°C and dispensed into 150 mm petri dishes. After incubation of enrichment samples, 10μL from each dilution replicate was placed on the surface of a spot plate and allowed to dry. Aliquots of host cultures were also placed on spot plates to check for viral contamination of host. Spot plates were incubated at 37°C for 24 hours. After incubation, dilution replicates were scored as positive or negative based on the presence or absence of lysis zones in or around the spots. Results for positive and negative spots were used to expressed concentrations as most probable number (MPN) of viruses per unit volume.

Data Analysis

Most probable number (MPN) calculations were done using the FDA Bacteriological Analytical Manual calculator (FDA, 2006). Data were analyzed using Excel 2003 (Microsoft, Redmond, WA) and Graph Pad Prism 5 (Graph Pad, San Diego, CA)

PPE experiments with human volunteers

Selection of Inoculum Size

In these experiments, the size of the inoculum used to contaminate PPE, including both the physical size of the inoculum used and the amount of virus contained in it, was chosen to approximate the characteristics of SARS virus inocula that might be naturally deposited on PPE during patient care. Because the main exposure route of concern in SARS transmission involves respiratory secretions, contamination of PPE might happen through patient activities such as coughing, or during droplet-producing patient care activities such as intubation. Therefore, experimental inoculum size and viral load should try to approximate that of respiratory secretions from humans infected with SARS. There are several reports in the literature on viral load in clinical specimens from SARS patients.

Most of these reports used quantitative RT-PCR methods, and viral loads are expressed in RNA copies/mL of sample. Wong *et al.* (2005) examined nasopharyngeal swabs of SARS patients and found that viral load ranged from 10^4 to 10^7 copies/mL, with a mean value of 10^7 copies/mL. Cheng *et al.* (2004) followed the pattern of viral shedding in SARS patients over time. They found that viral load in nasopharyngeal aspirates peaked around day 12-14 of infection, at 10^5 copies/mL. Their findings are similar to Hung *et al.* (2004) who found that mean viral load in nasopharyngeal aspirates around day 10–15 of infection was 10^5 copies/mL. This is lower than the range reported by Peiris *et al.* (2003), who found that peak viral load in nasopharyngeal aspirates of SARS patients around day 10 of infection ranged from 10^6 to 10^8 copies/mL. Estimates of the particle size of droplets expelled by people during activities such as breathing and coughing are difficult to find. One study of droplet size in the breath of healthy human subjects found that most droplets expelled are less than 1μm in size, and average about 83 droplets per liter of air expelled (Papineni and Rosenthal, 1997). This suggests that the total volume of droplets expelled by a person during coughing may be quite small. Based on this, virus inocula should be applied to PPE in a small volume of liquid to simulate possible contamination by droplets expelled by an infected individual. A volume of 25 μL is small, but enough that the fluorescent tracer is still visible. If the viral load in patient exudates is approximately 10^7 per mL, a 25μL volume would contain ~10^4 viruses. Therefore, a virus inoculum of approximately 10^4 total PFU of MS2 in 25 μL was used for these experiments.

Sample size calculations

Sample size for human subjects experiments was determined using the rule of zero for the probability of viral transfer from PPE. This can be used for a study design in which n

subjects are run through the protocol with a selected challenge input of viruses. In this design, n subjects undergo an experiment in which PPE is contaminated with a challenge inoculum of 10^4 organisms. The outcome is detectable transfer of virus (to other pieces of PPE, clothing, face, or hands). By determining how many of *n* subjects transfer virus, the probability that a random healthcare worker, when presented with this challenge, will transfer virus can be determined.

The confidence interval around this probability can also be determined. If, in a hypothetical experiment, it is found that none of the n subjects transfer any virus, it is known that the number of subjects who transfer virus is 0, and the probability that a random healthcare worker will transfer virus from PPE is 0. There is a distribution around this value, and the distribution has mean 0. The confidence interval around this mean of 0 can be calculated using the rule of zero, which says that for an experiment using n subjects, where 0 transfer virus, the upper bound of the 95% CI for the probability that a random healthcare worker, when presented with this challenge, will transfer virus is given by:

Upper bound of 95% CI = $1-[\exp(-2.9957/n)\}$

In a hypothetical experiment, 0/10 subjects transfer virus. Therefore:

$1-[\exp(-2.9957/10)\}= 0.25$

So, using 10 subjects, the confidence interval for the probability that random healthcare worker, when presented with this challenge, will transfer virus is 0%–25%. Therefore, if 0 of 10 subjects transfer virus, the actual probability that transfer will take place could be as low as 0% or as high as 25%. As the number of subjects grows, the confidence interval shrinks. For example, with 150 subjects, none of which transfer virus,

the upper limit of the confidence interval around the probability of zero is only 2%. Using 25% as the *a priori* acceptable upper limit for this confidence interval, the number of subjects chosen for the initial challenge was n=10.

Protocol for assessing transfer using human volunteers

The protocol for human volunteer experiments is shown in Figure 2-1. Protocols were approved by the UNC Biomedical IRB and written informed consent was obtained from volunteer participants. Enrolled participants met the following inclusion criteria: over 18, non-pregnant, non-latex-allergic, no active skin disorders, and medical evaluation approval for N95 respirator fit testing and use (OSHA, 1998). Experiments took place in a patient care room in the UNC Hospitals' General Clinical Research Center. Participants were shown the poster distributed by the CDC and given an opportunity to read it and ask questions. The poster was in front of the participant for reference while donning and removing PPE.

PPE (gowns, gloves, respirators, and goggles) donned by volunteers was contaminated with bacteriophage MS2, a non-enveloped, non-pathogenic RNA virus suspended in 0.01M PBS and GloGerm™ (GloGerm, Moab, UT), synthetic beads that fluoresce under ultraviolet light (for visual tracking of virus). Sites of contamination were: front shoulder of the gown, back shoulder of the gown, right side of the N95 respirator, upper right front of the goggles, and palm of the dominant hand. Each site was contaminated with a total of 10^4 plaque forming units (PFU) of MS2 in 5 drops of 5 μL each. Participants performed a healthcare task (measuring blood pressure on a mannequin) and then removed PPE according to the CDC protocol. Hands, items of PPE, and scrubs worn underneath were sampled for virus. Hands were sampled using the glove juice method (ASTM, 1994). Each hand was placed inside a bag containing 75 mL stripping solution

(0.4g KH2PO4, 10.1g Na2HPO4, 1.0 mL Triton-X /liter) and massaged for 60 seconds to cover all hand surfaces with solution. PPE items were immersed in 1.5% beef extract pH 7.5 and agitated on a reciprocal shaking platform at 120 cycles/min for 20 minutes. Eluent from hands and PPE was assayed for MS2 by most probable number (MPN) two-step enrichment method (EPA, 2001). To prevent cross-contamination, samples from only one volunteer were processed at a time, and individual eluent samples were processed separately in a biological safety cabinet, with decontamination in between.

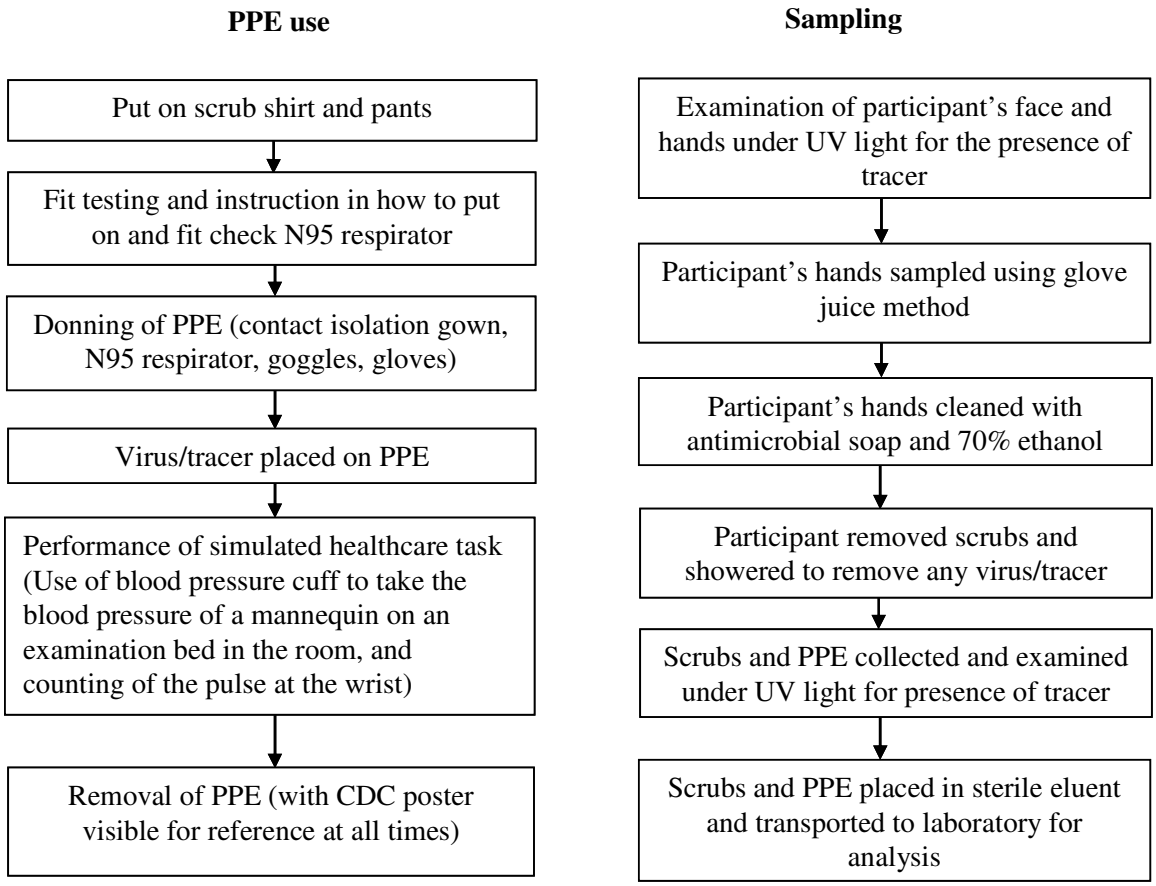

Figure 2-1. Protocol for human volunteer experiments assessing viral transfer from PPE

Effect of temperature and humidity on survival of surrogate coronaviruses.

Viral stocks were prepared as described above. Viral inocula were suspended in cell culture media to simulate the protein-containing matrices in which viruses may be deposited onto healthcare surfaces. Controlled humidity environments were created by the use of calcium sulfate granules (Drierite CO., Xenia, OH) or saturated salt solutions in sealed containers, and temperature and humidity were monitored using digital monitors. The test surfaces were 1 cm^2 stainless steel carriers with a No. 4 polish. Carriers were prepared by washing in 0.01% Tween 80, rinsing in 70% ethanol followed by distilled water, and autoclaving. Virus sample volumes of 10µL were inoculated onto three replicate carriers for each time point and placed in the controlled humidity environment. At each time point, carriers were removed, placed in a well plate, and covered with 1 mL 1.5% beef extract at pH 7.5. Viruses were eluted by gentle agitation at 60 RPM on a shaking platform for 20 minutes. The carrier was removed and the eluent assayed for infectivity on the appropriate cell line. Temperature and relative humidity conditions were:

- 4°C and 20% RH (Drierite)
- 4°C and 50% RH (saturated magnesium nitrate solution)
- 4°C and 80% RH (saturated sodium chloride solution)
- 20°C and 20% RH (Drierite)
- 20°C and 50% RH (saturated magnesium nitrate solution)
- 20°C and 80% RH (saturated ammonium chloride solution)
- 40°C and 20% RH (Drierite)
- 40°C and 50% RH (saturated magnesium nitrate solution)
- 40°C and 80% RH (saturated sodium chloride solution)

Other test surfaces used were glazed ceramic tile, laminate (Formica), plastic (polypropylene), contact isolation gowns (MediPak, Winchester, VA), latex gloves (Evolution One, Microflex, Reno, NV), respirators (N95 1860 healthcare particulate respirator, 3M Co., St. Paul, MN), nitrile gloves, and hospital scrub fabric. Carriers were 1 cm^2 pieces of the test surfaces. Fabric carriers were boiled for 10 minutes in reagent-grade water to remove any residual chemicals from laundering. Volumes of 10µL were inoculated onto three replicate carriers for each time point and placed in a controlled humidity environment at 20°C and 50% (±3%) to simulate the ambient conditions in healthcare environments. At each time point, carriers were removed, placed in a well plate, and covered with 1 mL 1.5% beef extract at pH 7.5. Viruses were eluted by gentle agitation at 60 RPM on a shaking platform for 20 minutes. The carrier was removed and the eluent assayed for virus infectivity on the appropriate cell line. Data analyses were carried out using Excel 2003 (Microsoft Corp.), and GraphPad Prism 5 (GraphPad, San Diego, CA). The parameter $\log_{10}(N_t/N_0)$ versus time was used to perform regression analysis on virus survival for each virus type, surface type, temperature, and relative humidity.

CHAPTER 3

Methods for the Recovery of a Model Virus from Healthcare Personal Protective Equipment

Introduction

Although nosocomial infections are a well-recognized risk for patients in many healthcare settings, healthcare workers are also affected. Caring for patients with communicable diseases places healthcare workers at risk for exposure to pathogens during patient care activities. Personal protective equipment (PPE) plays a crucial role in interrupting transmission of infectious bacterial and viral agents from patients to healthcare workers. PPE includes use of barriers (gowns, gloves, eye shields) and respiratory protection (masks, respirators) alone or in combination to protect mucous membranes, airways, skin, and clothing from contact with infectious agents (Siegel, 2007).

The importance of PPE in preventing healthcare worker infection was brought to the forefront by the worldwide outbreak of Severe Acute Respiratory Syndrome (SARS). The outbreak included a number of cases acquired by healthcare workers in the course of caring for SARS patients (Lau *et al.*, 2004; McDonald *et al.*, 2004), including medical students (Wong *et al.*, 2004), nurses (Loeb *et al.*, 2004), and emergency room personnel (Chen *et al.*, 2004). Studies of the spread of SARS in healthcare environments established a crucial role for PPE in preventing the spread of SARS from patients to healthcare workers.

The spread of SARS in healthcare facilities also brought renewed attention to the question of whether viral diseases can spread from person to person via fomites in the healthcare environment, as SARS-CoV nucleic acids were recovered from hospital surfaces in outbreak settings (Dowell *et al*., 2004). Furniture and healthcare equipment are not the only fomites in healthcare environments that have the potential to spread viruses. During the performance of healthcare tasks, PPE may become contaminated by viable pathogenic microorganisms. These microorganisms may be shed in patients' respiratory secretions, urine, or feces, or spread by contact, droplets, or aerosols from infected patients, and viruses can survive on the types of materials PPE is made from (Brady, Evans, and Cuartas, 1990, Bean *et al.*, 1982). Thus, PPE items are themselves types of fomites, and may play a role in the transmission of disease if they become contaminated with infectious viruses. This has been recognized by the Centers for Disease Control and Prevention (CDC) , which has a protocol outlining the proper sequence of removal of PPE items to minimize the risk of contamination to the wearer during removal (CDC, 2004).

The fate of contaminating microorganisms when PPE is removed and disposed of has important consequences for infection control, and the possibility that PPE itself may be a fomite that contributes to the spread of viruses such as SARS remains a poorly understood area in need of additional research. In order to determine the dynamics of virus survival and transmission via contaminated PPE and the attendant health risks, levels of viral contamination need to be quantified. Methods are needed to quantitatively recover viruses from items of PPE, including contact isolation gowns, N95 respirators, gloves, and eye protection. We describe methods for recovering a model virus,

bacteriophage MS2, from PPE. MS2 is a non-pathogenic, non-enveloped single-stranded RNA virus. Many of the viruses that have been identified as important causes of nosocomial viral infection or have high potential for nosocomial transmission, such as SARS-CoV, influenza, parainfluenza, mumps, measles, respiratory syncytial virus, and noroviruses, are single stranded RNA viruses, making MS2 a promising surrogate for the recovery of these viruses from healthcare PPE.

Materials and Methods

Preparation of virus stocks

Bacteriophage MS2 was propagated in the host bacterium *E. coli* C3000 (ATCC #15597) using the soft agar coliphage propagation method. Briefly, 50µL of virus stock was added to 30 mL of a log-phase host bacterial culture, grown on a rotating shaker for 4 hours at 37°C, and purified by chloroform extraction using a 2:1 volume ratio of virus to chloroform followed by centrifugation (5900×*g*, 30 minutes, 4°C). "Soft" tryptic soy top agar was prepared by adding agar to tryptic soy broth at a final concentration of 0.7%, and bottom agar plates were prepared using full strength TSA in 150 mm petri dishes. Purified virus stock (0.5 mL) and log phase host culture (0.5 mL) were added to 30 mL of soft agar and dispensed into bottom agar plates. Plates were incubated at 37°C for 24 hours. The top soft agar layer was then harvested with a pipette. Soft agar from all plates was pooled, purified by chloroform extraction as described above, and stored as stock in 20% glycerol-tryptic soy broth at -80°C.

Comparison of eluents for virus elution from PPE.

Virus stocks were diluted in 0.01M phosphate buffered saline, pH 7.2, to the desired concentration. Inocula for experiments were titered using the double agar layer (DAL)

plaque assay on tryptic soy agar (Becton Dickinson, Franklin Lakes, NJ) (EPA, 2001b). Virus was applied to 4 cm^2 swatches of contact isolation gown fabric in a single drop containing 10μL. Swatches were held at room temperature in a biological safety cabinet for 15 minutes, then immersed 250 mL sterile eluent solution. Eluent and PPE were agitated on a reciprocal shaking platform at 120 cycles/min for 20 minutes. Swatches were removed and discarded, and eluent was diluted in tryptic soy broth (TSB) (Becton Dickinson) and assayed for MS2 by the DAL method.

Elution of viruses from personal protective equipment

Virus stocks were diluted in 0.01M phosphate buffered saline to the desired concentration. For selected experiments, viruses diluted in PBS were monodispersed after dilution by sequential passage through 0.2 and 0.08 μm pore size filters (Millipore, Billerica, MA) pre-rinsed successively with 0.01% Tween 80 and sterile distilled water. Inocula for experiments were titered by DAL. Virus was applied to the surface of PPE in a single volume of 10μL. PPE items were held at room temperature in a biological safety cabinet for 15 minutes, and then immersed in 1-2 L eluent solution (depending on size of item). Eluent and PPE were agitated on a reciprocal shaking platform at 120 cycles/min for 20 minutes. PPE was removed from the eluent, the additional eluent was expressed into the container by wringing, and the item was discarded. The eluent was assayed for infectious virus using a two-step enrichment procedure for MS2 (EPA, 2001a).

PPE worn by human volunteers

Protocols for human volunteer experiments were approved by the UNC Biomedical IRB and written informed consent was obtained. Enrolled participants met the following inclusion criteria: over 18 years of age, non-pregnant, non-latex-allergic, no active skin disorders, and medical evaluation approval for N95 respirator fit testing and use.

Experiments took place in a patient care room in the UNC Hospitals' General Clinical Research Center. Volunteers donned contact isolation gowns (MediChoice, Arden, NC), gloves (Evolution One, Microflex, Reno, NV), respirators (N95 1860 healthcare particulate respirator, 3M Co., St. Paul, MN) and splashproof plastic goggles (Monogoggle, American Allsafe, Tonawanda, NY). Items of PPE were then contaminated with MS2 suspended in 0.01M PBS and with GloGerm™ (GloGerm, Moab, UT), synthetic beads that fluoresce under ultraviolet light (for visual tracking of virus). Sites of contamination were: front shoulder of the gown, back shoulder of the gown, right side of the N95 respirator, upper right front of the goggles, and palm of the dominant hand. Each site was contaminated with a total of 10^4 plaque forming units (PFU) of MS2, in with 5 drops of 5 μL each. Participants simulated a routine healthcare task by measuring blood pressure on a mannequin and then removed PPE. Gowns, N95 respirators, gloves, and goggles were collected after removal, immersed in eluent solution, and transported immediately back to the laboratory for analysis. Eluent and PPE were agitated on a reciprocal shaking platform at 120 cycles/min for 20 minutes. PPE was removed from the eluent, the additional eluent was expressed into the container by wringing, and the item was discarded. Eluent was assayed for infectious virus using the two-step enrichment method.

Two-step enrichment method

Eluent samples were diluted as needed using additional sterile eluent. For enrichment, 4M $MgCl_2$ (12.5 mL/L) and 10X tryptic soy broth were added to the final sample dilutions at a 1:20 (V/V) ratio of broth to sample. Appropriate dilutions were split into 10 replicate volumes per dilution, and 0.5 mL of log phase bacterial host was added to each replicate volume. Enrichment samples were incubated for 24 hours at 37°C. Host

bacterium cultures were also incubated at 37°C to check for contamination. For spot plates, 20 mL log phase bacterial host was added to 1L half-strength tryptic soy molten agar (30g tryptic soy broth, 7.5g Bacto agar per liter) at 45°C and dispensed into 150 mm petri dishes. After incubation of enrichment samples, 10µL from each dilution replicate was placed on the surface of a spot plate and allowed to dry. Aliquots of bacterial host cultures were also placed on spot plates to check for viral contamination of host. Spot plates were incubated at 37°C for 24 hours. After incubation, dilution replicates were scored as positive or negative based on the presence or absence of lysis zones within or around the spots. Results of positive and negative enrichment-spot plate volumes were expressed as most probable number (MPN) of viruses per unit volume of sample.

Data Analysis

Most probable number calculations were done using the FDA Bacteriological Analytical Manual calculator (FDA, 2006). Data on virus recoveries were statistically analyzed using Excel 2003 (Microsoft, Redmond, WA) and Graph Pad Prism 5 (Graph Pad, San Diego, CA)

Results

In the first phase, nine candidate eluent solutions were compared for their efficiency in eluting 5.8 $\log_{10}$ virus from 4 cm^2 swatches of contact isolation gown material. Beef extract was chosen based on its efficacy in recovering viruses from surfaces in other applications, such as ionically charged filters (Polaczyk, Roberts, and Hill, 2007). The effects of beef extract concentration (1.5% vs. 3%), pH (7.5 vs. 9.0), and the addition of 0.1% Tween 80, a detergent, were evaluated. PBS with 0.1% Tween 80 was also used to evaluate the effect of detergent alone. Virus stocks used in these initial experiments were

not monodispersed. Eluent samples were assayed by DAL. Virus recoveries using each eluent are shown in Table 3-1 and Figure 3-1.

Table 3-1. Evaluation of candidate eluents for recovery of MS2 from swatches of contact isolation gown material (original inoculum 5.8 $\log_{10}$ PFU)†

Eluent	% recovery (st. dev)
1.5% beef extract pH 7.5	73.0 (37.9)
1.5% beef extract pH 9.0	67.8 (49.2)
3% beef extract pH 7.5	63.9 (44.4)
3% beef extract pH 9.0	60.7 (41.8)
1.5% beef extract pH 7.5+ 0.1% Tween 80	52.5 (36.2)
1.5% beef extract pH 9.0+0.1% Tween 80	21.0 (24.1)
3% beef extract pH 7.5+0.1% Tween 80	20.1 (9.6)
3% beef extract pH 9.0+0.1% Tween 80	17.1 (14.2)
PBS+0.1% Tween 80	14.7 (8.7)

† average of 4 trials

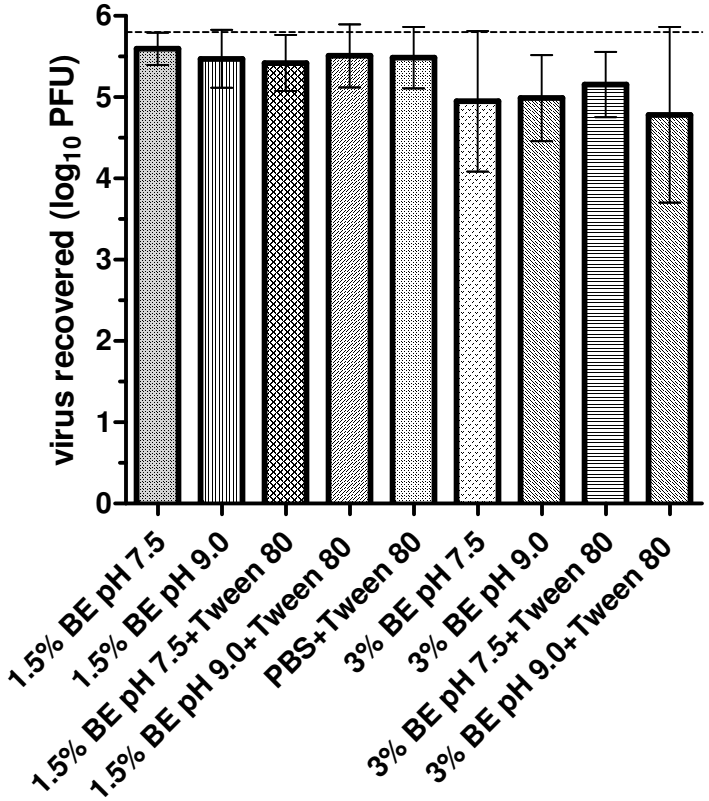

Figure 3-1. Evaluation of candidate eluents for recovery of MS2 from swatches of contact isolation gown material (average of 4 trials; dashed line: original inoculum 5.8 log_{10} PFU; bars: 95% CI)

No significant difference was found in recoveries from any of the nine eluents using one-way analysis of variance (ANOVA) (p=0.14). Four eluents with the highest mean recovery were then tested for their efficiency in eluting low numbers of virus (1.8 log_{10} PFU) from contact isolation gown swatches. To maximize recovery of low numbers of virus, the entire volume of eluent was examined using the two-step enrichment method. The results are shown in Figure 3-2.

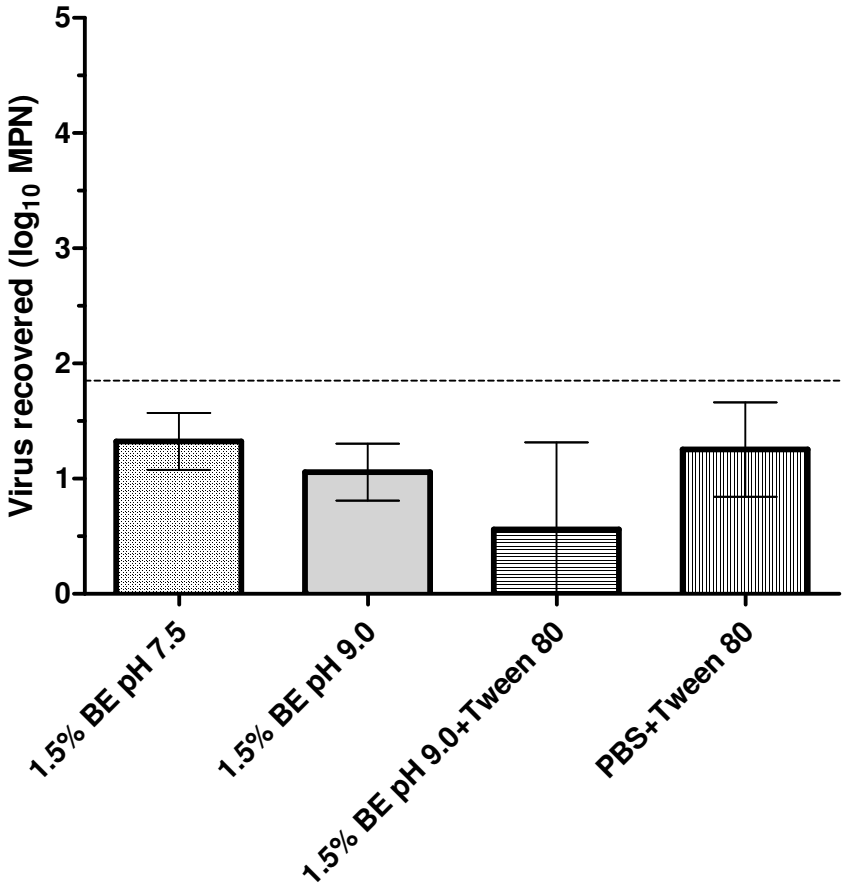

Figure 3-2. Evaluation of candidate eluents for recovery of MS2 from swatches of contact isolation gown material (average of 4 trials; dashed line: original inoculum 1.8 log_{10} PFU; bars: 95% CI)

The two eluents with the highest mean recovery, 1.5% beef extract pH 7.5 (mean 1.3 log_{10} MPN) and PBS+0.1% Tween 80 (mean 1.25 log_{10} MPN), did not differ significantly (unpaired *t* test, p=0.59). The solution with the simplest composition, 1.5% beef extract at pH 7.5, was chosen for subsequent experiments. This eluent was evaluated for its efficiency in eluting low numbers of MS2 (1.5 log_{10} PFU) from multiple PPE items using two-step enrichment assays of recovered elution fluid. PPE items tested were 4 cm^2 swatches of contact isolation gown fabric, whole contact isolation gowns, whole N95 respirators, splashproof goggles, and whole latex gloves. Gown swatches were immersed

in 250 mL eluent. PPE items were immersed in 1-2 L of eluent, depending on the size of the item. Results are shown in Figure 3-3.

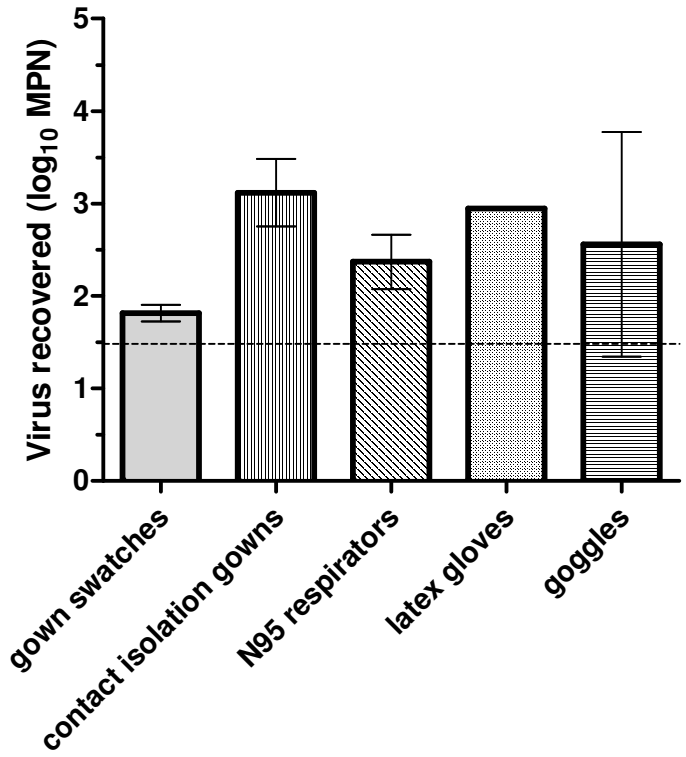

Figure 3-3. Recovery of MS2 from PPE using 1.5% beef extract pH 7.5 (dashed line: original inoculum 1.5 $\log_{10}$ PFU; bars: 95% CI)

Recovery from multiple PPE types using this eluent was variable, and was significantly greater than the inoculum titer. It was hypothesized that this was a result of viruses in the inoculum existing as aggregates. The titer of viruses existing in an aggregated state can be underestimated by plaque count methods due to single plaques being formed by an aggregate consisting of multiple viruses (Teunis, 2005). Protein solutions such as beef extract can disrupt aggregates formed by viruses (Gerba, 1984). If aggregated viruses in the inoculum were subsequently dispersed by the beef extract

eluent, the titer of virus recovered from the eluent would be higher than that of the inoculum. In an effort to address this problem, viruses in subsequent experiments were monodispersed by sequential filtration before being inoculated onto PPE. To evaluate the effect of dispersion, monodispersed MS2 was inoculated onto multiple PPE types in amounts of 1.5, 2.5, or 4.6 $\log_{10}$ PFU and eluted as described above. As seen in Figure 3-4, when virus is dispersed, the titer of virus recovered from gowns and other PPE types does not differ significantly (using unpaired t-test) from the inoculum titer, indicating that monodispersion of viral inocula prior to application is necessary to accurately measure viral recovery from experimentally contaminated PPE materials.

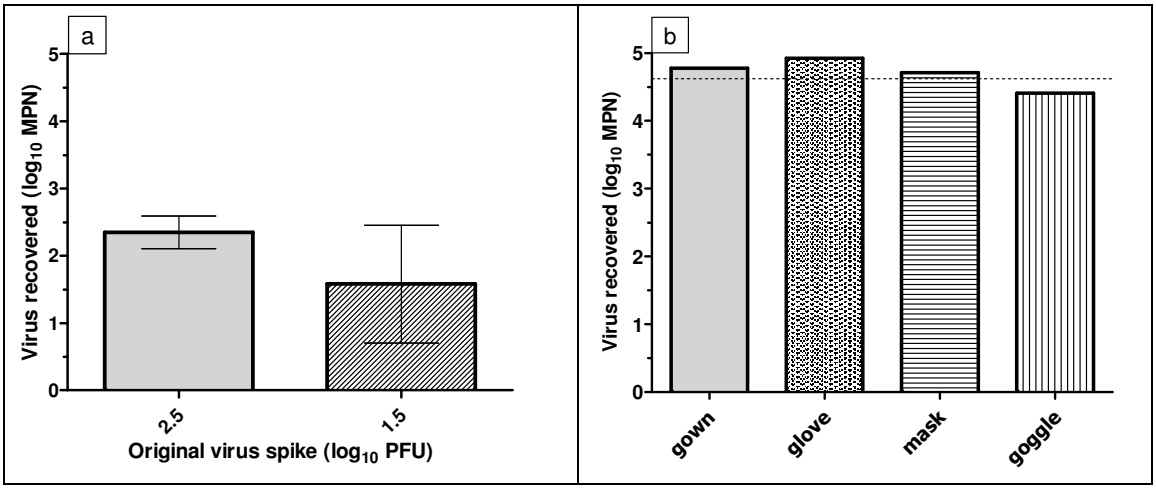

Figure 3-4. (a) Recovery of monodispersed MS2 from contact isolation gowns using 1.5% beef extract pH 7.5 (3 trials; bar: 95% CI) (b) Recovery of monodispersed MS2 from multiple PPE types using 1.5% beef extract pH 7.5 (2 trials; dashed line: inoculum 4.6 $\log_{10}$ PFU)

Based on the results of these experiments, two-step enrichment using beef extract eluent was then applied to items of PPE that underwent simulated viral contamination while being worn by human volunteers during a routine healthcare task. Ten subjects put on gowns, N95 respirators, gloves, and goggles, which were then contaminated with 4.3 $\log_{10}$ PFU of monodispersed MS2 (see Materials and Methods). After a volunteer took a

blood pressure on a mannequin to simulate a routine task that might be performed while wearing PPE, their PPE was removed and analyzed for recovery of MS2.

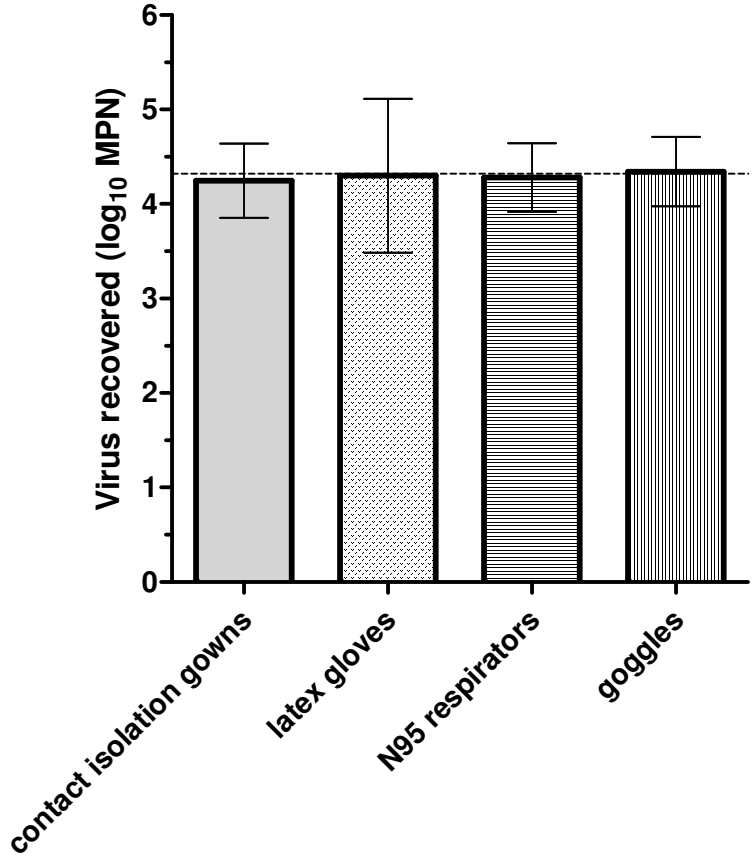

Figure 3-5. Recovery of MS2 from PPE worn by human volunteers during healthcare tasks (10 trials; dashed line: inoculum 4.6 $\log_{10}$ PFU; bars: 95% CI)

As shown in Figure 3-5, elution with 1.5% beef extract pH 7.5 followed by two-step enrichment assay can efficiently recover infectious MS2 from contaminated PPE that has been worn during the performance of a healthcare task. Recovery did not differ significantly among PPE types (one-way ANOVA, p=0.98).

Discussion

There are still knowledge gaps in assessing the risk of viral disease transmission posed by handling contaminated PPE. Accurate measurement of levels of viral contamination and the extent of viral survival on PPE items is vital information for such risk assessments. The existing literature on viral survival on PPE encompasses only some materials, and studies use a variety of methods to recover viruses from test materials, making comparisons between studies difficult. Different methods for viral recovery may have different recovery efficiencies, especially if the method was not specifically developed for the recovery of viruses. Viruses deposited on PPE in matrices of urine, feces, and respiratory secretions will likely be within a matrix consisting of water, salts and organic molecules. Viruses in such matrices behave as colloids, and their attachment to surfaces is largely governed by electrostatic and Van der Waals interactions with other molecules and surfaces (Gerba, 1984). These relationships at the molecular level may help to explain attachment of viruses to materials used in PPE as well; PPE items are often made of synthetic polymers that have different surface properties with respect to type and magnitude of charge and hydrophobic-hydrophilic properties. Examples are listed in Table 3-2.

Table 3-2. Characteristics of materials used in PPE

PPE	Materials	Polymer structure	Charge properties
Contact isolation gowns (Ellis, 2005)	polypropylene, polyethylene	carbon and hydrogen	hydrophobic
Eye protection	polyvinyl chloride	chlorine molecules	polar groups
	Polycarbonate	oxygen molecules	polar groups
Gloves	latex	vinyl acetate, styrene-butadiene, acrylates	polar or nonpolar groups
	polyvinyl chloride	chlorine molecule	polar groups
	polyurethane	oxygen molecules	polar groups
	nitrile	nitrile groups	polar groups
N95 respirator	microfibers	electrostatically charged	polar groups

As seen in Table 2, PPE can be made of molecules with both charged and hydrophobic characteristics. The lipid or protein outer coats of viruses have a net positive or negative surface charge depending on the pH of their surrounding environment; in neutral pH environments they tend to be negative. However, the protein coats of non-enveloped viruses can have pockets of hydrophobicity, and enveloped viruses have hydrophobic lipid membranes (Gerba, 1984). Therefore, virus attachment to PPE may be mediated by electrostatic interactions, when charged viral surfaces encounter charged groups on PPE surfaces, with the exact relationship depending on the pH of the surrounding matrix. It can also be mediated by hydrophobic reactions, when hydrophobic molecules on the surface of both viruses and PPE are excluded by the surrounding water molecules. Methods to elute viruses from PPE materials can be designed to disrupt these interactions. Robust and efficient virus recovery methods based on altering the adsorption and attachment behavior of viruses in contact with surfaces have been developed for eluting viruses from charged filter media using protein solutions (Polaczyk, Roberts, and Hill,

2007). Molecules in proteinaceous eluents have a variety of surface charges, and compete with viruses for attachment and adsorption sites on surfaces (Gerba, 1984).

This study demonstrated that a protein-based eluent, beef extract, is effective for eluting viruses from a range of PPE materials, possibly due to the disruption of both charged and hydrophobic interactions between the virus and the surface. Beef extract may also be efficacious for the elution of other types of viruses from PPE, including enveloped viruses, but this topic needs further research.

These virus recovery methods can be used to expand our knowledge of viral survival on PPE. To date, there have been few studies assessing the survival of pathogens on materials used to make personal protective equipment (Yassi *et al*., 2005). Although there have been studies of bacterial survival on N95 respirators (Wang, 1999), no such studies exist for viruses. Some investigations have found that viruses can survive on materials used to make other types of PPE. When deposited in high numbers (~10^6 $TCID_{50}$), SARS-CoV has been found to survive on gowns for up to 2 days (Lai, Cheng, and Lim, 2005). Enveloped ssRNA viruses have been shown to survive on latex glove material. For example, human coronavirus 229E can survive for up to 2 hours, although it loses up to 85% of its infectious titer (Sizun *et al*., 2000), and avian influenza virus can survive for up to 6 days without loss of infectious titer (Tiwari *et al*., 2005). Non-enveloped RNA viruses such as human rotavirus and hepatitis A virus can survive for several days on latex under ambient conditions, with only ~1 $\log_{10}$ loss in infectious titer (Abad, Pinto, and Bosch, 1994). Viruses deposited on PPE are likely to be associated with respiratory secretions, urine, or feces, and viruses, which may enhance viral survival. SARS Co-V, for example, can retain its infectivity for hours in feces and days in respiratory secretions

(Lai, Cheng, and Lim, 2005). These existing studies suggest that viruses have the potential to survive on PPE materials for longer than single-use PPE is usually worn, creating the potential for viral transfer when PPE is handled after wearing. Pittet *et al* (2006) described five steps necessary for pathogens to be transferred from patient to patient via healthcare workers' hands (Figure 3-6):

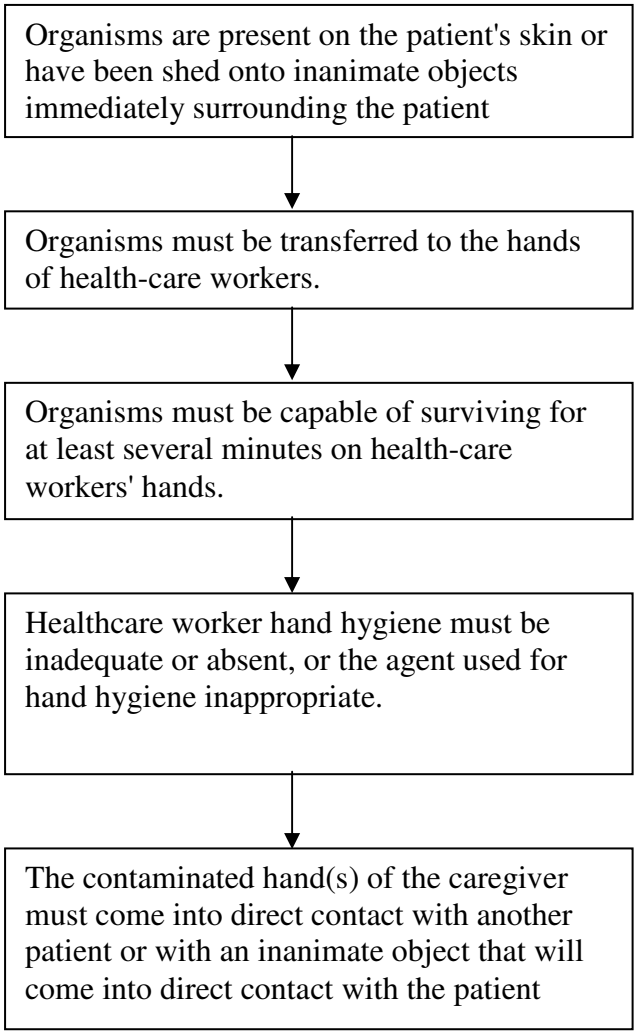

Figure 3-6. Sequence of events for transfer of pathogens from patient to patient via healthcare workers' hands (from Pittet *et al*., 2006).

Contaminated PPE can play a role in steps 1-2. If patients shed viruses onto healthcare workers' PPE in the course of patient care, these viruses can remain infectious when PPE

is removed. Transfer of viruses from experimentally contaminated fabrics (Rusin, Maxwell, and Gerba, 2002), plastic surfaces (Gwaltney and Hendley, 1982), and gloves (Hall, Douglas, and Geiman, 1980) to hands has been demonstrated, suggesting that viruses can transfer from PPE to hands when contaminated items are handled in the course of removal and disposal. In addition, contamination can be present on skin after exposure to pathogens even when PPE is worn (Zamora *et al*., 2006), and may be transferred to used items of PPE if they are handled after removal. Virus transfer between hands and PPE items can encourage both accidental autoinoculation by the healthcare worker and subsequent transmission of viruses to other patients, staff, or family members, especially when inadequate hand hygiene is practiced (Pittet *et al*., 2006).

Viral contamination of PPE items also has important implications if PPE is reused. Availability of PPE is recognized as an important factor affecting proper use (Moore *et al.,* 2005). Although reuse of disposable PPE for multiple patient encounters is not recommended (Siegel *et al*., 2007), there may be situations in which PPE is in short supply and reuse is difficult to avoid. Reuse could be a serious concern in outbreak settings with high patient loads, or in low-resource settings where the cost of single-use disposable PPE is prohibitive. Reuse of PPE such as gloves, though discouraged (WHO, 2006) takes place in under-resourced healthcare settings (Gunasekera *et al*., 1997; Mbanya, Ateudjieu, and Kaptue, 1998). During the SARS outbreak, there may have been shortages of PPE items in some facilities (Farquharson and Baguley, 2003; Lau *et al*., 2004; Lee, Wee, and Johan, 2005; McDonald *et al*., 2004); under these circumstances, items of PPE may be reused for multiple patient encounters (Shaw, 2006). If PPE is used, handled, and reused during multiple patient encounters, there are many potential

opportunities for the spread of viruses from contaminated items to the face, hands, and body of the user, as well as from patient to patient via the healthcare worker. Robust methods to measure viral contamination on PPE items can be applied to more accurately assess these and other microbial risks of PPE removal, handling and reuse in all healthcare settings. The methods described here are the first that have been shown to give high virus recovery with multiple types of PPE. These methods can be used to conduct rigorous studies of viral survival on PPE and virus transfer to and from PPE for risk assessments in infection control and healthcare worker protection.

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CHAPTER 4

Assessing the Risk of Viral Transfer from Contaminated Personal Protective Equipment to Employees' Skin and Clothing in the Healthcare Setting

Introduction

Caring for patients with communicable diseases places healthcare workers (HCWs) at risk. Infected HCWs may suffer serious illness or death, and may spread infection to other HCWs, their families, or patients. Methods to prevent HCW infections include vaccination (CDC, 1997; Weber and Rutala, 2008), hand hygiene (Boyce and Pittet, 2002), and isolation of patients with communicable diseases (Siegel *et al*., 2007).

A key aspect of patient isolation is proper use of personal protective equipment (PPE) to protect HCWs from pathogen exposure during patient care. PPE includes use of barriers (gowns, gloves, eye shields) and respiratory protection (masks, respirators) to protect mucous membranes, airways, skin, and clothing from contact with infectious agents (Siegel *et al*., 2007). The importance of PPE was underscored in the outbreak of Severe Acute Respiratory Syndrome (SARS). HCWs, accounted for approximately 20% of cases, and developed SARS while performing patient care (Chan-Yeung *et al*., 2004, Lau *et al*., 2004a). Studies demonstrated that failure to properly use PPE was a risk factor for HCW infection (Lau *et al*., 2004b).

This outbreak raised concern that HCWs could contaminate their skin or clothes with pathogens during PPE removal, resulting in accidental self-inoculation as well as subsequent virus spread to patients, other HCWs, or fomites. The Centers for Disease

Control and Prevention (CDC) addressed this by designing a protocol to minimize wearer contamination during the PPE removal process (Figure 4-1) (CDC, 2004). However, the effectiveness of this protocol in preventing self-contamination has not been validated.

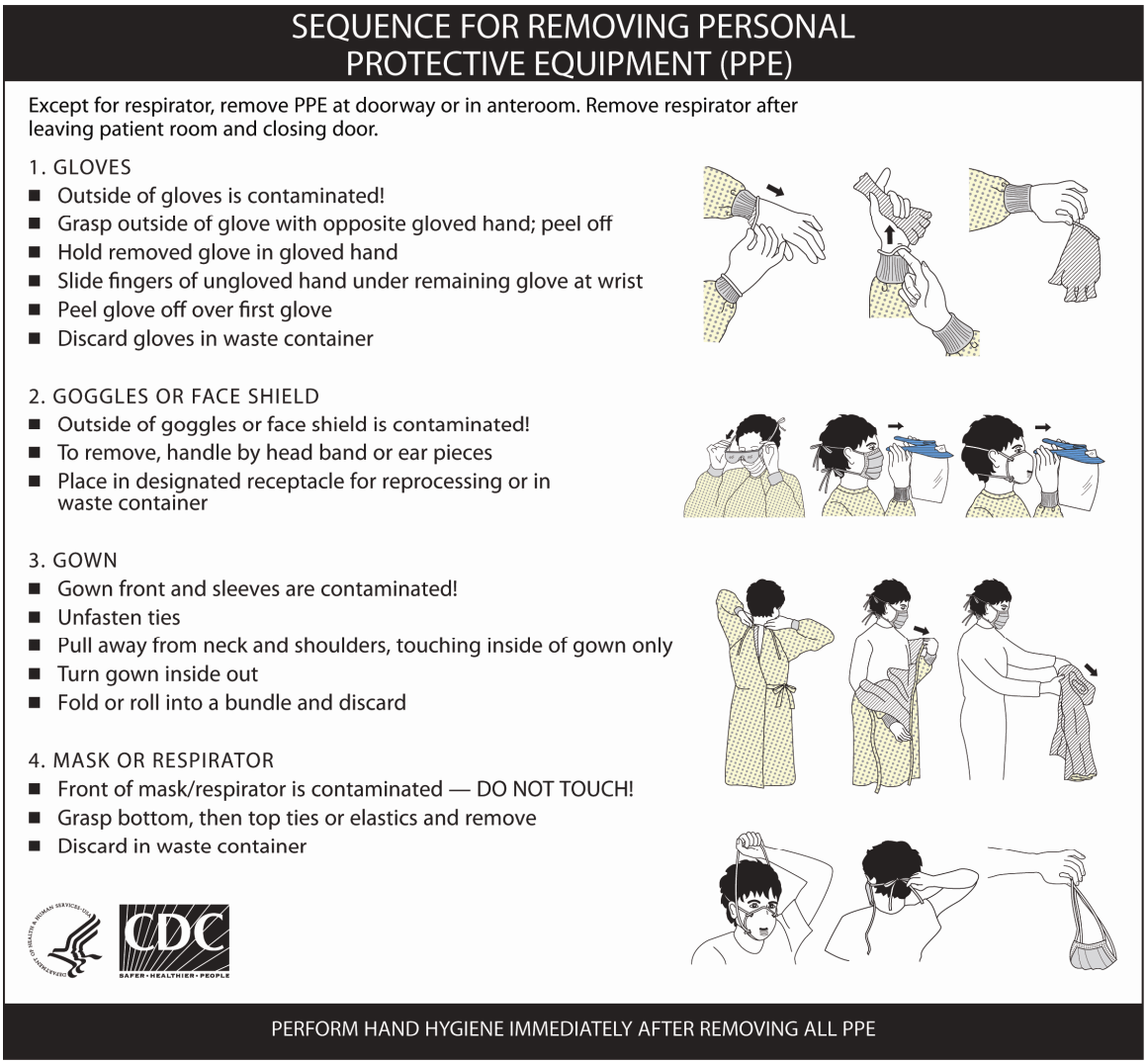

Figure 4-1. CDC protocol for removing healthcare worker PPE

By examining the fate of viruses after removal of experimentally contaminated PPE under controlled conditions, it can be determined if removing PPE according to the CDC protocol prevents viral contamination of the wearer. A human challenge study was

undertaken using a non-pathogenic virus to determine the fate of viruses on items of PPE when a wearer removes PPE in accordance with the CDC protocol.

Methods

PPE (gowns, gloves, respirators, and goggles) donned by volunteers was contaminated with bacteriophage MS2, a non-enveloped, non-pathogenic RNA virus suspended in 0.01M PBS and GloGerm™ (GloGerm, Moab, UT), synthetic beads that fluoresce under ultraviolet light (for visual tracking of virus). Sites of contamination were: front shoulder of the gown, back shoulder of the gown, right side of the N95 respirator, upper right front of the goggles, and palm of the dominant hand. Each site was contaminated with a total of 10^4 plaque forming units (PFU) of MS2 in 5 drops of 5 μL each. Participants performed a healthcare task (measuring blood pressure on a mannequin) and then removed PPE according to the CDC protocol. Hands, items of PPE, and scrubs worn underneath were sampled for virus. Hands were sampled using the glove juice method (American Society for Testing and Materials, 1994). Each hand was placed inside a bag containing 75 mL stripping solution (0.4g KH_2PO_4, 10.1g Na_2HPO_4, 1.0 mL Triton-X /liter) and massaged for 60 seconds to cover all hand surfaces with solution. PPE items were immersed in 1.5% beef extract pH 7.5 and agitated on a shaker for 20 minutes. Eluent from hands and PPE was assayed by most probable number (MPN) enrichment infectivity assay (EPA, 2001). To prevent cross-contamination, samples from only one volunteer were processed at a time, and individual eluent samples were processed separately in a biological safety cabinet, with decontamination in between.

Using an *a priori* value of 25% for the 95% upper confidence limit when p(transfer)=0, the sample size was n=10. Protocols were approved by the UNC Biomedical IRB and

written informed consent was obtained. Enrolled participants met the inclusion criteria: over 18, non-pregnant, non-latex-allergic, no active skin disorders, and medical evaluation approval for N95 respirator fit testing and use (OSHA, 1998). Experiments took place in a patient care room in the UNC Hospitals' General Clinical Research Center. The experimental protocol is shown in Figure 4-2. Participants were shown the poster distributed by the CDC (Figure 4-1) and given an opportunity to read it and ask questions. The poster was in front of the participant for reference while donning and removing PPE.

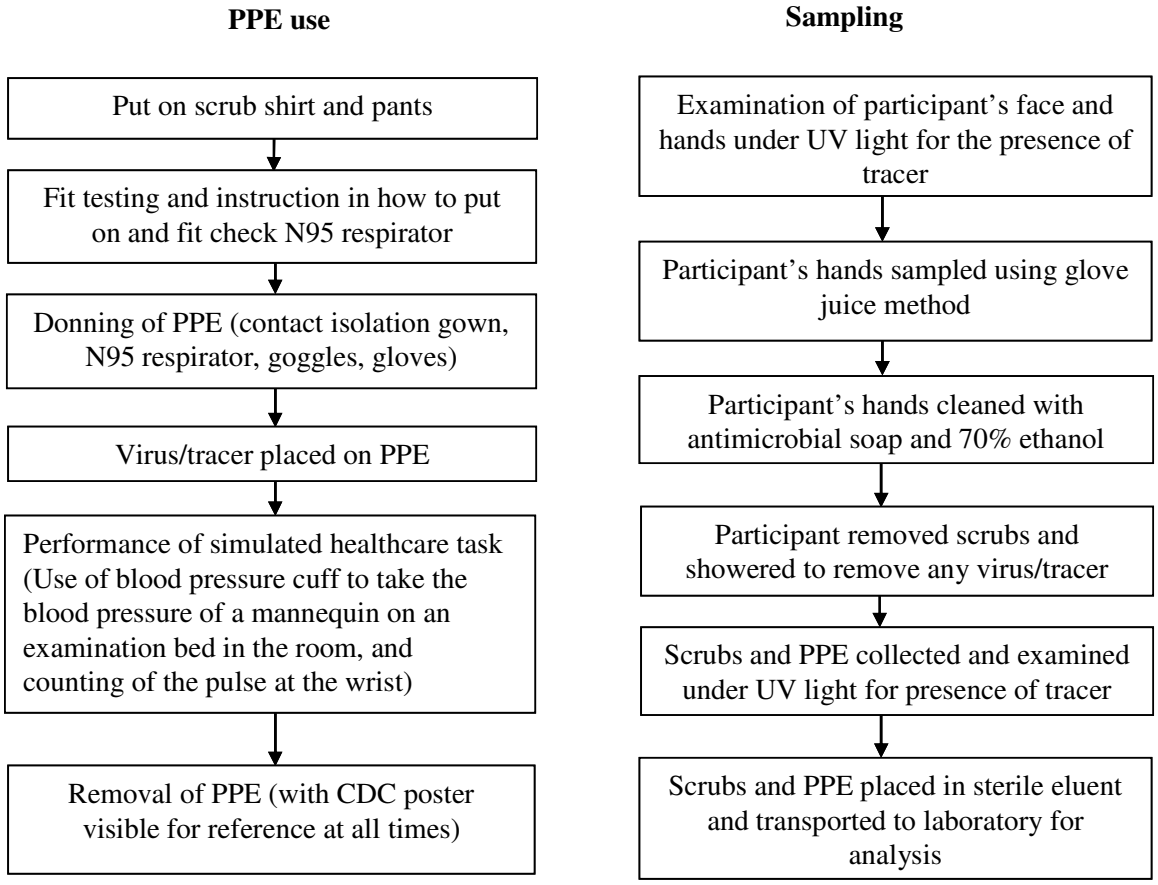

Figure 4-2. Protocol for human challenge experiments

Results

Ten subjects participated in this study; nine females and one male. Nine subjects were right-handed and one left-handed. Transfer of virus to both hands, the initially uncontaminated glove on the non-dominant hand, and the scrub shirt and pants worn underneath the PPE was observed in most volunteers (Table 4-1). Due to the difficulty of sampling large facial areas, the presence of visible fluorescent tracer was used as the criterion to determine whether the face would be sampled. No tracer was observed on the facial areas of any volunteer. The fluorescent tracer was not a consistent indicator of virus contamination, with virus recovered both from sites where tracer was visible and where it was not detected.

Table 4-1. Frequency and Levels of Viral Contamination of Selected Sites

Site	Percent of volunteers who transferred virus to site (N=10)	Mean viral titer recovered from site (log10 MPN)	Proportion of contaminated sites with visible tracer (N=10)
Non-dominant glove	80%	2.2	10%
Right hand (skin)	90%	2.4	20%
Left hand (skin)	70%	1.8	0%
Scrub shirt	100%	3.2	10%
Scrub pants	75%*	2.1	0%
Face	0%	--	--

*N=8

The amount of virus recovered ranged from 1-3 $\log_{10}$ MPN for hands and 1-4 $\log_{10}$ MPN for scrubs. The mean amount of virus recovered from the right hand (the dominant hand of 9/10 volunteers) was greater than that recovered from the left hand. While removal of gloves and gowns required two hands, mask and goggle removal was one-handed, which could have resulted in larger quantities of virus transferred to the

dominant hand during removal. In the single left-handed subject, recovery of virus was greater from the left hand than the right (1.82 vs. 0.98 $\log_{10}$ MPN). The mean amount of virus recovered from scrub shirts was significantly greater than that recovered from pants (p=0.01), possibly due to contact with hands when the gown is pulled away from the shoulder during removal.

Discussion

PPE is vital for protecting HCWs from occupationally acquired infection during patient care, particularly droplet- or airborne-transmitted diseases (Weber and Rutala, 2008). However, removing PPE after patient care without contaminating skin or clothes is important. PPE is usually worn only for short periods, while viruses such as influenza (Bean *et al*., 1982) and SARS-CoV (Rabenau *et al*., 2005) can survive for hours on surfaces, and viral infection can be spread by surface-to-hand (Gwaltney and Hendley, 1982) and hand-to-hand contact (Gwaltney *et al*., 1978).

Developing and validating an algorithm for the removal of PPE that prevents contamination of the skin and clothes of HCWs is key to interrupting nosocomial transmission of potentially serious infectious agents, including SARS, avian influenza, hemorrhagic viruses and poxviruses. These experiments showing viral transfer demonstrate that the current CDC algorithm is insufficient to protect HCWs from contamination during PPE removal, but several potential options exist that might prevent such contamination, including double gloving, use of surgical protocols for PPE removal, and PPE impregnated with an antimicrobial agent.

A double glove removal sequence would begin with removal of the outer glove, followed by goggles or face shield, gown, and respirator/mask, and finishing with

removal of the inner glove followed by hand hygiene. Using this method, handling of PPE with ungloved hands is avoided. The use of an inner glove ½ size larger than usually worn may be used to improve dexterity and reduce constriction when double gloving. Borrowing PPE protocols from surgery, where the ends of gown sleeves are tucked underneath gloves during wear, might also reduce contamination. When finished, goggles and respirator are removed first, and gown and gloves are then removed together by peeling off both at the same time, again avoiding handling PPE with ungloved hands. Finally, the use of PPE impregnated with antimicrobial agents might also reduce or eliminate contamination of skin and clothes.

This study also indicates the need for continued emphasis on hand hygiene. A barrier to improving hand hygiene compliance rates is the belief that gloves make hand hygiene unnecessary (Pittet *et al*., 2001). This is contradicted by our study and others showing that organisms can spread from gloves to hands after glove removal (Doebbeling, *et al*., 1988). Even if double gloving is incorporated into protocols for PPE use, it is vital to emphasize that it is not a substitute for proper hand hygiene. Before these or other candidate methods are introduced into clinical practice, their impact on the safety of HCWs should be validated by testing using methods such as we have described.

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CHAPTER 5

Survival of Surrogate Coronaviruses in Water

Introduction

Members of the family *Coronaviridae* have been recognized for many years as a cause of common-cold like, self-limiting respiratory infections in humans (Monto, 1997). With the emergence of Severe Acute Respiratory Syndrome (SARS) in a worldwide outbreak in 2003, there was new recognition that coronavirus infection could result in serious, even fatal disease. The agent of SARS was quickly found to be a previously unrecognized coronavirus, named SARS coronavirus (SARS-CoV) (Drosten *et al*., 2003). In the age of global travel, large healthcare facilities, and high density housing developments, SARS CoV was novel not only because it was a newly emerged human pathogen of the *Coronaviridae* family, but for the routes by which it appeared to spread in human populations. A respiratory pathogen transmitted from person-to-person by droplets and aerosols, SARS-CoV spread from patients to healthcare workers in emergency rooms and other healthcare facilities (Chen *et al*., 2004, Seto *et al*., 2003), from patients to visitors in a hospital (Varia *et al*., 2003), and from passenger to passenger on an airplane (Olsen *et al*., 2003). When an outbreak of SARS occurred in Amoy Gardens, a large apartment complex in Hong Kong, it was found that SARS-CoV shed in the feces an infected individual visiting one of the buildings may have spread via viral aerosols that entered the bathrooms of other apartments through faulty toilet plumbing and floor drains, transmitting SARS to other occupants of the building

(McKinney *et al*., 2006). Because of this unique fecal-droplet-respiratory route, it is possible that during outbreaks, water contaminated with the fecal waste of infected individuals becomes subsequently aerosolized to possibly serve as a vehicle of transmission for SARS. In order to better assess the risks from this exposure pathway, data are needed on the survival and persistence of SARS-CoV in water and sewage. If SARS-CoV is found to be capable of surviving for relatively long periods of time in water, a droplet route of exposure and transmission would be supported as plausible.

Because SARS requires specially trained personnel working in BSL-3 laboratory containment, there are significant challenges involved in gathering such data, and very little data are currently available. However, other members of the *Coronaviridae* family may provide representative survival data that can be used to conduct risk assessments of SARS. In order to determine the survival and persistence of coronavirus infectivity in water and sewage over time, two animal coronaviruses were studied for survival in different water types at two ambient temperatures as possible surrogates for the survival of SARS-CoV.

The family *Coronaviridae* is divided into three groups. Groups I and II include human, mammalian, and avian coronaviruses, and Group III consists of avian coronaviruses. Although SARS is thought to be related to the Group 2 coronaviruses (Jackwood, 2006), and phylogenetic analyses have indicated it may be closely related to mouse hepatitis virus (MHV) (Lio and Goldman, 2004), there is still disagreement about the exact placement of SARS-CoV within the coronavirus family (Gorbalenya, Snijder and Spaan, 2004). Based on this uncertainty, one representative of each group of mammalian coronaviruses was included in the study to determine if they differed in their survival and

persistence in water. The two viruses studied were transmissible gastroenteritis virus (TGEV), a diarrheal pathogen of swine and a member of the Group I coronaviruses, and mouse hepatitis virus (MHV), a pathogen of laboratory mice and a member of the Group II coronaviruses (Jackwood, 2006). The survival of these two viruses in reagent-grade water, lake water, and settled human sewage was observed over a period of weeks to provide estimates of how long members of the coronavirus family, as surrogates of SARS-CoV, may remain infectious in these waters.

Materials and methods

Test waters

Reagent-grade water was produced from laboratory tap water by a Dracor™ water purification system (Dracor, Durham, NC) which includes reverse osmosis and ultraviolet light treatment. Lake water came from University Lake, an impoundment that serves as the drinking water source for the town of Chapel Hill, NC ,and was obtained from the raw water inlet of the Orange Water and Sewer Authority (OWASA) drinking water treatment plant. Settled sewage was obtained from the OWASA waste water reclamation facility, and was pasteurized in a waterbath at 70°C for 3 hours to inactivate other microorganisms that would interfere with cell culture infectivity assays of coronaviruses.

Preparation of viral stocks

TGEV and MHV were kindly provided by R. Baric, University of North Carolina, Chapel Hill. TGEV was grown in swine testicular (ST) cell cultures. MHV was grown in delayed brain tumor (DBT) cell cultures. Viral stocks were propagated by infecting confluent layers of host cell cultures in flasks, harvesting cell lysates, clarifying by centrifugation (3000×g, 30 min, 4°C), and storing resulting supernatants as virus stock at

-80°C. Viral titers were determined by quantal assays for CPE and expressed as the most probable number (MPN) method. Assays were in confluent host cell layers in 24-well plates containing maintenance medium consisting of Eagle's minimum essential medium (MEM), 10% bovine serum replacement (Fetal Clone II, Hyclone, Logan, UT), 10% lactalbumin hydrolysate and gentamicin (0.1 mg/mL)/kanamycin (0.05 mg/mL) and nystatin (75 U/mL).

Survival experiments

For each virus, 5 mL of clarified virus stock was spiked into duplicate 45 mL aliquots of test water. A positive control sample for measuring the initial virus concentration in water at time 0 was taken and assayed immediately after spiking. One aliquot of test water was held at room temperature (23-25°C), and another one was held at refrigerator temperature (4°C). At each time point, samples were taken and assayed for virus infectivity on the appropriate host cell line. Four replicate samples were assayed at each time point. Virus survival at each time point was expressed as $\log_{10}(N_t/N_0)$, where N_t is the virus concentration in MPN/mL at time *t*, and N_0 is the initial virus concentration in MPN/mL in the positive control sample at time 0.

Statistical Analysis

Analysis was carried out using Excel 2003 (Microsoft Corp.), and GraphPad Prism 5 (GraphPad, San Diego, CA). The parameter $\log_{10}(N_t/N_0)$ versus time was used to perform regression analysis for each virus and water type. Coefficients from regression analysis were used to predict times needed for 90, 99, 99.9, and 99.99% reduction of each virus at each temperature in test waters.

Results

The change in infectious titer of TGEV and MHV in reagent-grade water (pH 6.0, turbidity 0.6 NTU) over 49 days at 4°C and 25°C is summarized in Figure 5-1 (a) and (b). Observed viral reduction values are plotted as individual data points. Predicted viral reduction values obtained from regression analysis are plotted as lines (MHV, black lines, TGEV, gray lines). There was a progressive decline in the infectivity of both TGEV and MHV over 49 days at 25°C, and the reduction in infectivity of both viruses at 25°C follows typical first-order kinetics (Fig 1a). Reductions of both viruses at 25°C as determined by regression analysis (as $\log_{10} N_t/N_0$) are shown in Table 5-1.

Table 5-1. Reduction of TGEV and MHV infectivity ($\log_{10} N_t/N_0$) in reagent-grade water at 25°C

Day	TGEV	MHV
0	0	0
7	-0.6	-0.8
14	-1.2	-1.6
19	-1.7	-2.2
29	-2.6	-3.3
43	-3.8	-4.8
49	-4.4	-5.5

The infectivity of TGEV declined by approximately 0.6 $\log_{10}$ per week, and infectious MHV declined by approximately 0.8 $\log_{10}$ per week. Times for 99% reduction in infectious titer in RGW at 25°C was 22 days for TGEV and 17 days for MHV. Comparison of regression lines at 25°C for TGEV and MHV showed a significant difference in inactivation rates between the two viruses at this temperature ($p<0.001$). As shown in Figure 5-1(b), there was no significant decline in infectious titer of either virus over 49 days at 4°C.

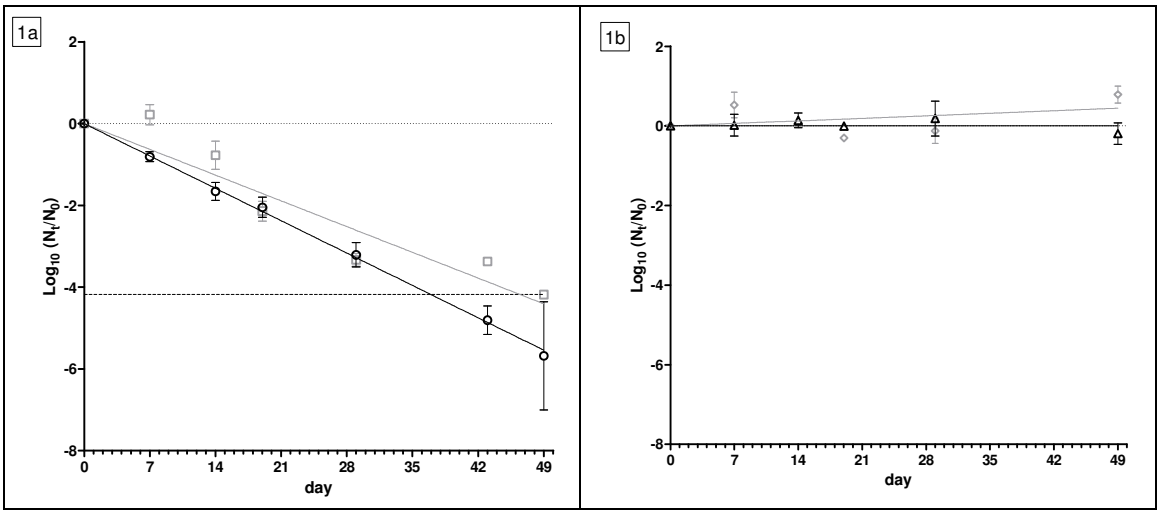

Figure 5-1. Infectivity of TGEV and MHV $\log_{10}$ (N_t/N_0) over 49 days in reagent-grade water, 4 trials per plotted time point. Observed data=individual points; predicted values from regression analysis=continuous lines. (a) Infectivity at 25°C (gray squares and lines=TGEV; black circles and lines=MHV; dashed line=TGEV detection limit) (b) Infectivity at 4°C (gray diamonds and lines=TGEV; black triangles and lines=MHV)

Figure 5-2 shows the infectious TGEV and MHV titers in lake water (pH 7.5, turbidity 1.73 NTU) over 14 days at 4°C and 25°C. Time required for 99% reduction in infectious titer in lake water at 25°C was 13 days for TGEV and 10 days for MHV. At 4°C, TGEV infectivity declined by approximately 1 $\log_{10}$ by day 14; in contrast, MHV infectivity persisted with no decline in titer after 14 days at 4°C. Because there were only 2 time points (7 and 14 days), regression analysis was not performed on data from lake water.

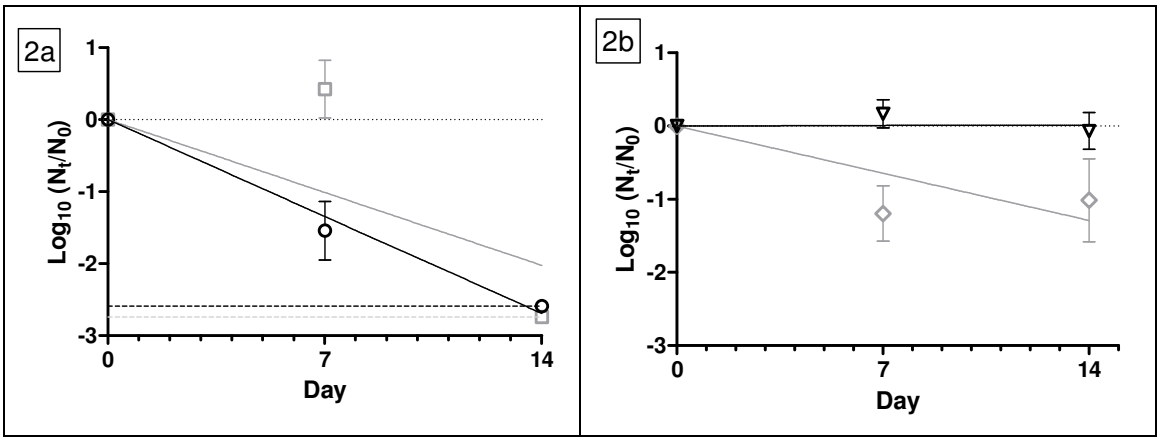

Figure 5-2. Infectivity of TGEV and MHV as $\log_{10}$ (N_t/N_0) over 14 days in lake water, 4 trials per point. Observed data=individual points; predicted values from regression analysis=continuous lines. (a) Infectivity at 25°C (gray squares and lines=TGEV; black circles and lines=MHV; dashed lines=detection limits) (b) Infectivity at 4°C (gray diamonds and lines=TGEV; black triangles and lines=MHV)

The change in infectious titer of TGEV and MHV in pasteurized settled sewage (pH 7.6, turbidity 17.6 NTU) over 35 days at 4°C and 25°C is summarized in Figure 5-3. Observed viral infectivity reduction values are plotted as individual data points. Predicted viral infectivity reductions obtained from regression analysis are plotted as solid lines (MHV, black lines, TGEV, gray lines). Both viruses exhibited a similar slow rate of decline in infectivity in settled sewage at 4°C, and a few percent of the initial viruses were still detectable after 35 days. Regression analysis showed that TGEV declined by approximately 0.3 $\log_{10}$ per week, and MHV by 0.2 $\log_{10}$ per week. Comparison of regression lines at 4°C for TGEV and MHV showed that inactivation rates for the two viruses differed significantly (p=0.01).

There was a progressive decline in the infectivity of both TGEV and MHV over 35 days at 25°C. The reduction in infectivity of both viruses at 25°C follows typical first-order kinetics (Fig 3a). There was a more rapid decline in infectivity titer of both viruses at 25°C than at 4°C (the experiment at 25°C was terminated at day 21 due to subsequent

growth of contaminating microorganisms in the test water). Regression analysis showed that infectivity of TGEV at 25°C declined by approximately 1.5 $\log_{10}$ per week, and that of MHV declined by approximately 2 $\log_{10}$ per week. Times for 99% reduction in infectious titer were 9 days for TGEV and 7 days or MHV. Comparison of regression lines at 25°C for TGEV and MHV showed that inactivation rates differed significantly between the two viruses (p=<0.001).

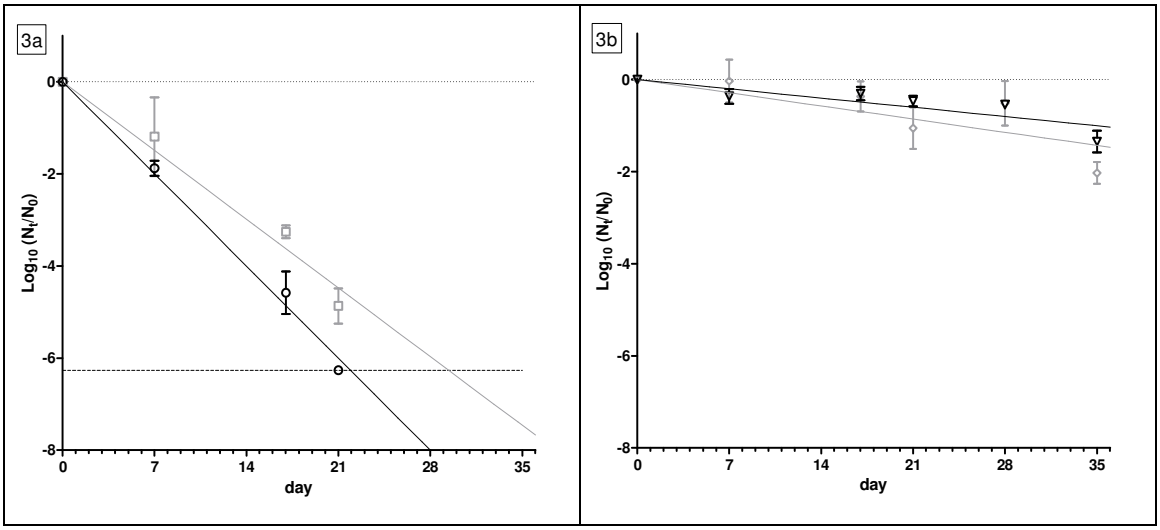

Figure 5-3. Infectivity of TGEV and MHV as $\log_{10}$ (N_t/N_0) over 35 days in pasteurized settled sewage, 4 trials per point. Observed data=individual points; predicted values from regression analysis=continuous lines. (a) Infectivity at 25°C (gray squares and lines=TGEV; black circles and lines=MHV; dashed line=MHV detection limit) (b) Infectivity at 4°C (gray diamonds and lines=TGEV; black triangles and lines=MHV)

Regression analysis on data from reagent-grade water and pasteurized settled sewage was compared to determine if water quality has an effect on virus survival. The results summarized in Table 5-2 show the predicted values obtained by regression analysis to achieve 90%, 99%, 99.9%, and 99.99% reduction of TGEV and MHV in each water type.

Table 5-2. Predicted times for decimal reductions of TGEV and MHV infectivity in different water types at 4°C and 25°C

	Reagent grade water				Pasteurized settled sewage			
Reduction	25°C		4°C		25°C		4°C	
$\log_{10}(N_t/N_0)$ (%)	TGEV	MHV	TGEV	MHV	TGEV	MHV	TGEV	MHV
-1 (90%)	11	9	110	>365	4	3	24	35
-2 (99%)	22	17	220	>365	9	7	49	70
-3 (99.9%)	33	26	330	>365	14	10	73	105
-4 (99.99%)	44	35	330	>365	19	14	98	139

The time required for 4 $\log_{10}$ (99.99%) infectivity reduction of TGEV at 25°C is longer in reagent-grade water than in pasteurized settled sewage (44 days vs. 19 days). This is also true of MHV infectivity (35 days in reagent-grade water vs. 14 days in sewage). There is also a difference in predicted inactivation times between viruses. In both water types, the predicted time to achieve a 4 $\log_{10}$ reduction in viral infectivity titer at 25°C is longer for TGEV than for MHV (44 days vs. 35 days in reagent-grade water and 19 days vs. 14 days in pasteurized settled sewage). At 4°C, the time required to achieve a 4 $\log_{10}$ reduction in infectivity titer in pasteurized settled sewage was 98 days for TGEV vs. 139 days for MHV, and predicted times for 4 $\log_{10}$ infectivity reduction of both viruses in reagent-grade water were approximately 1 year. Because viral titer declined so slowly at 4°C, regression analysis based on this data set (where the longest elapsed time was 49 days) may not be a reliable way to predict viral reduction over long periods at 4°C in these water types.

TGEV and MHV can survive and remain infectious for long periods in different water types ranging from highly treated water, to surface water to settled sewage at both low (4^oC) and at typical temperate (25°C) ambient temperatures. In all water types, the titer of infectious virus declined more rapidly at 25°C than at 4°C. The extent of virus reduction

also differed with water quality, with more rapid inactivation in reagent-grade water than settled sewage. In both reagent grade water and pasteurized settled sewage, inactivation rates were significantly different between the two viruses at 25°C. A linear regression model incorporating incubation time as a continuous variable and virus type (TGEV or MHV), water type (reagent grade water or pasteurized settled sewage) and temperature (4°C or 25°C) as dichotomous variables found that water type ($p=0.0071$), incubation time ($p<0.0001$) and temperature ($p<0.0001$) were significant predictors of $\log_{10}$ viral reduction. Virus type was not a significant predictor ($p=0.28$). Inclusion of an interaction variable for water type and temperature did not show significant interaction between these parameters ($p=0.47$).

Discussion

The extent of SARS-CoV survival in water, sewage and other aqueous media has been previously studied. Rabenau *et al*. (2005) found that the titer of SARS-CoV declines approximately 0.5 $\log_{10}$ over 9 days in a serum-free suspension of cell-culture medium at room temperature. This is a slower rate of inactivation than was observed for TGEV and MHV in reagent-grade water and pasteurized settled sewage, and may be due to a protective effect of the buffers, salts and organic nutrients found in cell culture medium. Longer survival in water with protective salts is supported by data from other investigators who found that SARS-CoV survived longer in PBS (14 days) than in dechlorinated tap water or domestic sewage (2 days) at 20°C (Wang *et al.,* 2005). This is a much shorter survival time than was demonstrated for TGEV and MHV in this present study, but the authors did not report the actual change in titer or detection limit of the

assays performed. Therefore, a quantitative comparison of inactivation rates of the different coronaviruses in these aqueous media is not possible.

At 4°C, the titer of infectious TGEV and MHV in all test water types remained stable over the course of the experiments. This is consistent with other investigations that found SARS-CoV persisting at least 14 days in domestic sewage and dechlorinated tap water at 4°C. Again, direct quantitative comparisons of inactivation rates are difficult as these investigators did not report the actual changes in viral titers over time (Wang *et al*., 2005). In the present study coronavirus infectivity in water and sewage was followed for longer time periods than in previous studies using SARS Co-V. The coronaviruses studied were capable of remaining infectious in clean, highly treated waters, natural environmental waters, and waters contaminated with human fecal waste for periods of weeks. Infectivity titer reductions after time periods of about 6 weeks ranged from none, to slight (<1 $\log_{10}$) to modest (1-2 $\log_{10}$) at 4°C, depending on water quality and virus type.

Temperature and incubation time were significant predictors of viral reduction, which is consistent with previous studies of viral survival in water (Yates, Gerba, and Kelley, 1985; Enriquez, Hurst, and Gerba, 1995). Water type was a significant predictor of the rate of viral reduction, with greater reduction seen in pasteurized settled sewage as compared to reagent grade water. There are several factors that could contribute to greater reduction in more contaminated water, including pH, chemical constituents, and the presence of other microorganisms. MHV is stable over a pH range of 5-7.4 at 37°C and 3-10 at 4°C (Daniel and Talbot, 1987), and TGEV is stable over a pH range of 5-7 at 37°C and 5-8 at 4°C (Pocock and Garwes, 1975). In pasteurized settled sewage spiked with MHV, pH declined over a period of weeks (data not shown). However, the pH

remained within the range of stability for these viruses, suggesting that it may not have be a significant factor in declining viral infectivity. This finding of a lack of pH effect on virus survival is consistent with those of previous studies (Yates, Gerba, and Kelley, 1985). Other investigations have found that virus survival in water is influenced by high molecular weight dissolved matter (Noble, and Fuhrman, 1997), which is present in higher concentrations in sewage. Chemical constituents found in sewage may also have antiviral activity (Sobsey *et al*., 1980); antiviral effects may be due to the activity of bacteria feeding on the viral macromolecules and producing metabolites having antiviral activity, such as proteolytic enzymes that attack virion proteins (Deng and Cliver, 1992; Deng and Cliver, 1995). Such antiviral activities of microbes in wastewater may be responsible for the lower rates of coronavirus survival observed in sewage than in reagent water in this study.

It has been well-established with other human pathogens that formation of droplets and aerosols from water contaminated with microorganisms can serve as a vehicle for transmission of both respiratory and enteric infections, including *Legionella* (Butler and Breiman, 1998), *Cryptosporidium* (CDC, 1998). and aerosolization of body fluids and fecal matter can serve as a source of both enteric and respiratory infections, such as noroviruses (Marks, 2003) and hantaviruses (LeDuc, 1998). SARS has demonstrated a unique human-to-human fecal-droplet-respiratory transmission route, observed in the Amoy Gardens apartment building SARS outbreak. When an individual shedding infectious virus in their feces used the toilet facilities in a building, a combination of faulty drain traps and the use of powerful exhaust fans in residential units resulted in virus-laden liquid droplets being drawn into living spaces via floor drains. The droplets

were inhaled by other occupants and carried on air currents to other areas of the building, causing an outbreak (McKinney *et al*., 2006; WHO, 2003). Although more data is needed on the survival of SARS-CoV in fecal droplets and aerosols to assess this risk pathway, the airborne fecal droplet transmission model, and the length of time that coronaviruses have been shown to remain infectious in water and sewage, suggest that aqueous media fecally contaminated by SARS-CoV could pose a health risk in future outbreaks.

The results of this study suggest that coronaviruses can survive for sufficiently long periods of time in water and sewage for these vehicles to serve as a source of exposure. The persistent survival of coronaviruses at low temperatures in highly treated potable waters has important exposure risk implications for its spread via fecally contaminated water should it re-emerge. If SARS-CoV-contaminated water or sewage becomes aerosolized, it could potentially cause virus exposure to large groups of people. This could be an ongoing risk during an outbreak, even in the presence of quarantine measures to isolate infected individuals from others until they are recovered. Building water or sewer systems contaminated with persistent infectious SARS coronavirus might also defeat quarantine measures by continuing to spread virus even after an infected individual has been removed from the area. The persistence of coronaviruses in water and sewage found in this study suggests that quarantine measures, which proved effective in containing the last SARS outbreak, could be seriously undermined unless adequate attention is paid to the safety and security of building plumbing systems. Should SARS or other fecally transmissible respiratory viruses emerge in the future, outbreak control measures should include adequate inspection, repair, and disinfection of water and sewerage systems in structures where outbreaks have taken place. Such measures can

ensure that if these systems become contaminated by fecal waste from infected individuals, they do not become water-related vehicles for the continued spread of disease, even after infected hosts are no longer present. Further experiments are necessary to better determine the kinetics of SARS-CoV survival or inactivation in water, sewage and other aqueous media. Presently, TGEV and MHV may serve as conservative indicators of the survival of SARS-CoV in water and sewage.

Conclusions

- TGEV and MHV can survive and remain infectious for long periods in different water types including highly treated water, surface water, and sewage
- TGEV and MHV can survive and remain infectious at both low (4^{o}C) and at typical temperate ambient (25°C) temperatures.
- In all water types, the titer of infectious virus declined more rapidly at 25°C than at 4°C.
- Water type, incubation time, and temperature were significant predictors of $\log_{10}$ viral reduction kinetics.
- TGEV and MHV may serve as conservative indicators of the survival of SARS-CoV in water and sewage.
- The persistent survival of coronaviruses at low temperatures in highly treated potable waters has important exposure risk implications for its spread via fecally contaminated water should it re-emerge in human populations.

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CHAPTER 6

Survival of surrogate coronaviruses on environmental surfaces

Introduction

The possible role of environmental surfaces in the spread of nosocomial viral infection has been recognized for some time (Sepkowitz, 1996; Sattar, 2004), with healthcare surface disinfection being proposed for the control of viruses such as influenza, rotavirus, calicivirus, and coronaviruses (Sattar, 2004). The question of whether hospital surfaces play a role in the spread of nosocomial viral infection took on particular urgency during the worldwide outbreak of severe acute respiratory syndrome (SARS), a novel coronavirus infection which was driven partly by nosocomial spread. Cases of SARS were documented among healthcare workers, patients, and visitors in healthcare facilities (McDonald *et al.*, 2004). During outbreaks of SARS in healthcare facilities, studies were done to determine whether SARS coronavirus (SARS-CoV) was present on surfaces in outbreak settings. Sampling of surfaces in hospitals in Taiwan and Thailand, including surfaces in patient rooms and areas on floors used for isolation of SARS patients, found SARS-CoV nucleic acids on surfaces and inanimate objects in patient rooms, nurses' stations, and public areas of the hospital (Dowell *et al.*, 2004, Booth *et al.*, 2005). Infectious virus was not cultured from any of the samples, so it is unclear whether this evidence of viral nucleic acid was indicative of infectious SARS-CoV presence on hospital surfaces in outbreak settings. The presence of nucleic acids, however, suggests the possibility that infectious virus was present on these surfaces for a period of time, and

these surfaces could become sources of further viral transmission. Assessing the risk posed by SARS-CoV on surfaces is difficult without knowing how long these viruses survive on environmental surfaces, and how that survival is affected by the type of surface and factors in the surrounding environment, such as temperature and humidity level. Gathering such data using SARS-CoV is difficult due to the challenges of working with this virus in biosafety level 3 containment.

The development of surrogates for studying the environmental survival of SARS-CoV would advance our understanding of the survival and resistance of this virus on environmental surfaces, the possible role of such surfaces in the transmission of SARS, and the risk posed by contaminated surfaces in outbreak settings. The objective of this work was to determine the effect of temperature and humidity on the survival of surrogate coronaviruses for SARS-CoV on surfaces.

The selection of coronaviruses as surrogates for SARS-CoV is complicated by the fact that genetic analysis suggests SARS-CoV is not a member of either of the two currently recognized groups of mammalian coronaviruses (Marra *et al*., 2003; Ruan *et al*., 2003). Therefore, to elucidate possible differences in survival among members of the Coronaviridae that are possible surrogates for SARS, viruses from both groups of mammalian coronaviruses were selected. Transmissible gastroenteritis virus (TGEV), an enteric swine pathogen, and 229E, a human respiratory pathogen, were selected form Group 1. Mouse hepatitis virus (MHV), a murine neurologic pathogen, and OC43, a human respiratory pathogen, were selected from Group 2. These candidate viruses were used to evaluate the survival of coronaviruses on different surface types, and the effect of temperature and humidity on coronavirus survival on these surfaces.

Materials and Methods

Preparation of viral stocks

TGEV and MHV were kindly provided by R. Baric, University of North Carolina, Chapel Hill, NC; 229E and OC43 were kindly provided by D. Erdman, Centers for Disease Control and Prevention, Atlanta, GA. Cell lines were obtained from the American Type Culture Collection (Manassass, VA) and the Lineberger Comperhensive Cancer Center (University of North Carolina Chapel Hill). Cell lines used were: swine testicular cells for TGEV (ST, ATCC# CRL-1746); delayed brain tumor cells (DBT) for MHV; human lung fibroblast cells for 229E (MRC-5, ATCC# CCL-171); human ileocecal colorectal adenocarcinoma cells for OC43 (HCT-8, ATCC# CCL-244). Viral stocks were propagated by infecting confluent layers of host cell cultures in flasks, harvesting cell lysates, clarifying by centrifugation (3000×g, 30 min, 4°C), and storing resulting supernatants as virus stock at -80°C. Viral titers were determined by quantal assays on confluent host cell layers in 24-well plates and computed by the most probable number (MPN) method.

Infectivity assays

TGEV and MHV were propagated and assayed in maintenance medium consisting of Eagle's minimum essential medium (MEM), 7.5% sodium bicarbonate, HEPES buffer, 10% bovine serum replacement (Fetal Clone II, Hyclone, Logan, UT), 10% lactalbumin hydrolysate, non-essential amino acids, 1 mM sodium pyruvate and gentamicin (0.1 mg/mL)/kanamycin (0.05 mg/mL). 229E and OC43 were propagated and assayed in maintenance medium consisting of Eagle's minimum essential medium (MEM), 10% newborn calf serum (Fetal Clone II, Hyclone, Logan, UT), 7.5% sodium bicarbonate,

HEPES buffer, glutamine, non-essential amino acids, 1 mM sodium pyruvate, gentamicin (0.1 mg/mL)/kanamycin (0.05 mg/mL) and nystatin (100 U/mL).

For TGEV, MHV, and 229E assays, inoculated cell cultures were examined microscopically after incubation for visible cytopathic effect. Coronavirus OC43 does not produce visible cytopathic effect when grown in HCT-8 cells. To measure the infectivity of this virus, primers were designed targeting the O (open reading frame 1) genes for use in combined cell culture infectivity/reverse transcriptase polymerase chain reaction (RT-PCR) assays. Confluent layers of HCT-8 cells were infected in 24-well plates and incubated for 5 days. Inoculated cell layers were washed 2x with PBS to remove residual virus from the cell layer. Viral RNA was then extracted from cells using the RNEasy Mini Kit (Qiagen, Valencia, CA). Viral RNA was detected using RT-PCR with the Qiagen OneStep RT-PCR kit in a Gradient Cycler. Nucleic acid was amplified in a 25μL reaction containing 5μL of RNA template, 5μL 5x Qiagen 1-step RT-PCR buffer, 0.2μM dNTP, 0.6μM each primer, 10U RNasin RNase inhibitor (Promega, Madison, WI), and 1μL Qiagen OneStep RT-PCR Enzyme Mix.

Primers orfw (5´-CGGGATCCATGAGAACGGTGATAAATTAGATCAGT-3´) and orfrt (5´-GCACACGACTACCTTCTACATCAAATG-3´) were used to amplify a 156 bp fragment of the ORF1 gene. Conditions were: Reverse transcription at 50°C, 30 min; activation at 95°C 15 min; 40 cycles of denaturation at 94°C 30s, annealing at 50°C 1 min, and extension at 72°C 1.5 min; and a final extension step of 72°C for 10 min. DNA was visualized on 2% agarose gels with ethidium bromide staining. Heat-inactivated OC43 (65°C, 60 min) was used as a control to check for false-positive PCR signals from inactivated virus.

Survival experiments

Viral stocks were prepared as described above. Viral inocula were suspended in cell culture media to simulate the protein-containing matrices in which viruses may be deposited onto healthcare surfaces. Controlled humidity environments were created by the use of calcium sulfate granules (Drierite CO., Xenia, OH) or saturated salt solutions in sealed containers, and temperature and humidity were monitored using digital meters. For temperature and humidity experiments, test surfaces were 1 cm^2 stainless steel carriers with a No. 4 polish. Carriers were prepared by washing in 0.01% Tween 80, rinsing in 70% ethanol, followed by rinsing in distilled water, and then autoclaving. Sample volumes of 10µL were inoculated onto three replicate carriers for each time point and inoculated carriers placed in the controlled humidity environment. Control carriers (time 0 samples) were sampled immediately after drying at the desired temperature and humidity level. At each time point, carriers were removed, placed in a well plate, and covered with 1 mL 1.5% beef extract 7.5. Viruses were eluted by gentle agitation on a shaking platform for 20 minutes. The carrier was removed and the eluent was assayed for virus infectivity on the appropriate cell line.

Other test surfaces used were ceramic tile, laminate (Formica), plastic (polypropylene), contact isolation gowns (MediChoice, Arden, NC), latex gloves (Evolution One, Microflex, Reno, NV), respirators (N95 1860 healthcare particulate respirator, 3M Co., St. Paul, MN), and nitrile gloves. Carriers were 1 cm^2 pieces of the test surfaces. Sample volumes of 10µL were inoculated onto three replicate carriers for each time point and placed in a controlled humidity environment at 20°C and 50% (±3%) to simulate the ambient conditions in healthcare environments. At each time point, carriers were removed and eluted as described above. Data analysis was carried out using Excel 2003

(Microsoft Corp.), and GraphPad Prism 5 (GraphPad, San Diego, CA). The parameter $\log_{10}(N_t/N_0)$, (where N_t is the virus concentration in MPN at time *t*, and N_0 is the initial virus concentration in MPN on the positive control sample at time 0) versus time was used to perform regression analysis on virus inactivation rates for each test condition of virus type, temperature, and relative humidity.

Results

Eluent comparison

Four candidate eluents were tested for their efficiency in eluting TGEV and MHV from the stainless steel carriers used in experiments: PBS, TSB, 1.5% beef extract pH 7.5, and cell culture medium (CCM). Virus was placed on carriers in 10µL volumes and allowed to dry in a biological safety cabinet, eluted, and the eluate assayed for viral infectivity. Results are shown in Figure 6-1. The quantity of initial virus recovered did not differ significantly by eluent (p=0.31) (the amount of virus applied to carriers was 4.39 $\log_{10}$ PFU for MHV and 3.25 $\log_{10}$ PFU for TGEV). The mean recovery of both viruses was slightly higher using 1.5% beef extract pH 7.5. This was selected as the eluent for subsequent experiments.

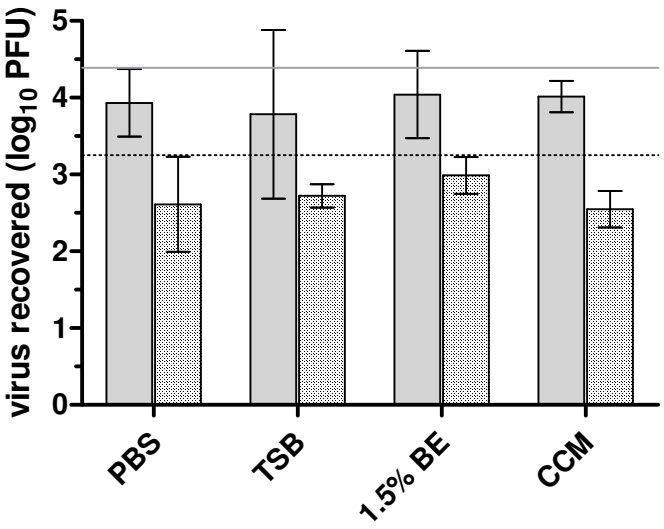

Figure 6-1. Comparison of eluents for the recovery of TGEV and MHV dried onto stainless steel carriers. Gray bars, MHV (solid gray line: virus titer deposited on carrier, 4.39 $\log_{10}$); dotted bars, TGEV (dashed line: virus titer deposited on carrier, 3.25 $\log_{10}$).

Temperature and humidity

The survival of TGEV, MHV, and 229E was determined at three temperatures: 4°C, 20°C and 40°C. For each temperature, three relative humidity (RH) levels were evaluated: 20%, 50%, and 80% RH. The effect of humidity on viral survival at 4°C is shown in Figure 6-2.

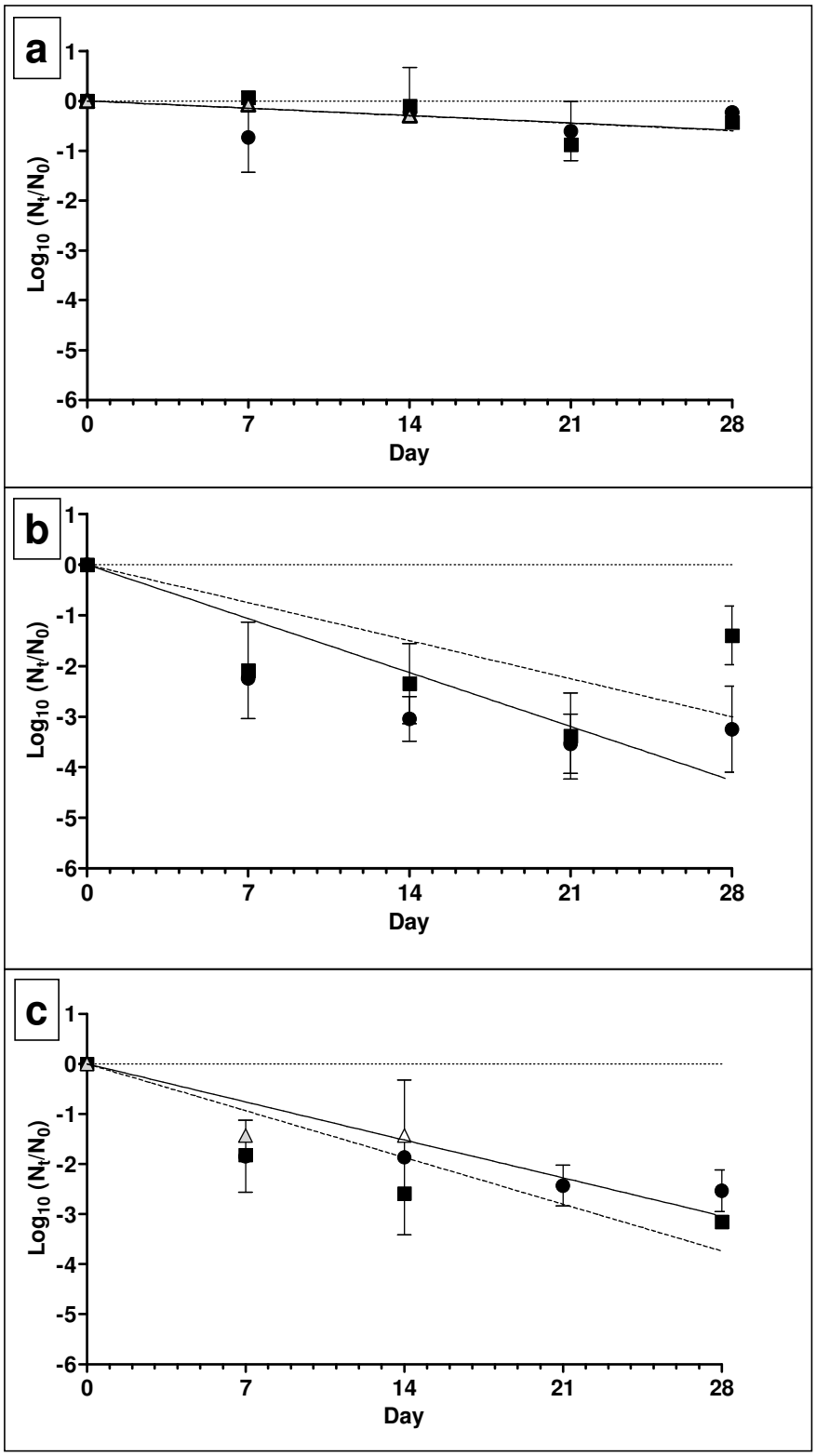

Figure 6-2. Survival of TGEV, MHV, and 229E at 4°C a) 20% RH b) 50% RH c) 80% RH. (Black squares: TGEV; black circles: MHV; gray triangles: 229E; symbols with white centers: sample was below detection limit of the assay; solid line: regression line for TGEV; dashed line: regression line for MHV)

Viral titer was stable for up to 28 days at 4°C and low relative humidity, with loss of less than 1 $\log_{10}$ of initial infectious virus titer over 14 days for 229E at 20% and 80% RH (experiment was terminated at 14 days) and over 28 days for TGEV and MHV. At 50% RH, 229E did not survive drying onto the carrier. The decline in TGEV and MHV infectivity titer follows first-order (log-linear) kinetics. The slopes of regression lines for TGEV and MHV at 4°C and each RH level are shown in Table 6-1.

Table 6-1. Slopes of regression lines for virus inactivation rates at 4°C and RH levels of 20, 50 and 80%

	Slope of regression line (95% CI)		
% RH	TGEV	MHV	229E
20	-0.021 (-0.033 to -0.01)	-0.021 (-0.034 to -0.0082)	-0.019 (-0.036 to -0.0012)
50	-0.11 (-0.14 to -0.07)	-0.15 (-0.18 to -0.12)	Too few points to estimate
80	-0.13 (-0.16 to -0.11)	-0.11 (-0.13 to -0.09)	-0.11 (-0.14 to -0.083)

Comparing the slopes of the regression lines, the rate of change in infectious titer (inactivation rate) of TGEV at 20% RH is significantly different from that at 50% and 80% RH. However, inactivation rates of TGEV at 50% and 80% RH do not differ significantly from each other. The inactivation rates of MHV are significantly different at all three humidity levels. The inactivation rates for 229E at 20% and 80% RH also differ significantly from each other. At each humidity level, the virus inactivation rates (slopes of regression lines) do not differ significantly among the three viruses. Inactivation at 50% and 80% RH was more rapid than at 20% RH over the period of the experiment. Rates of virus inactivation are sufficiently low that infectious viruses deposited on surfaces in high numbers (3 to 4 $\log_{10}$ viruses) could persist for 28 days at all three RH levels (low, medium and high) and low temperature.

The effect of humidity on viral survival at 20°C is shown in Figure 6-3. Inactivation was more rapid at 20°C at all humidity levels compared to 4°C However, TGEV and MHV could survive for weeks at 20% RH, with a 2 $\log_{10}$ decline in infectious titer over 28 days. At 50% RH, both TGEV and MHV infectious titer declined by 2.3 $\log_{10}$ in 3 days. At 80% RH, TGEV infectivity titer declined by 3.1 $\log_{10}$ in 14 days; at 50% RH, a 5 $\log_{10}$ decrease in TGEV infectivity titer, as predicted by regression analysis, would take place in 5 days. MHV infectivity declined by 5 $\log_{10}$ in 10 days at 80% RH, compared to a predicted decline of 5 $\log_{10}$ in 7 days at 50% RH. 229E was inactivated more rapidly, with infectivity declining by 1.2 $\log_{10}$ within 24 hours at 20% RH and by 0.6 $\log_{10}$ within 24 hours at 80% RH. 229E did not survive drying onto the carrier at 50% RH.

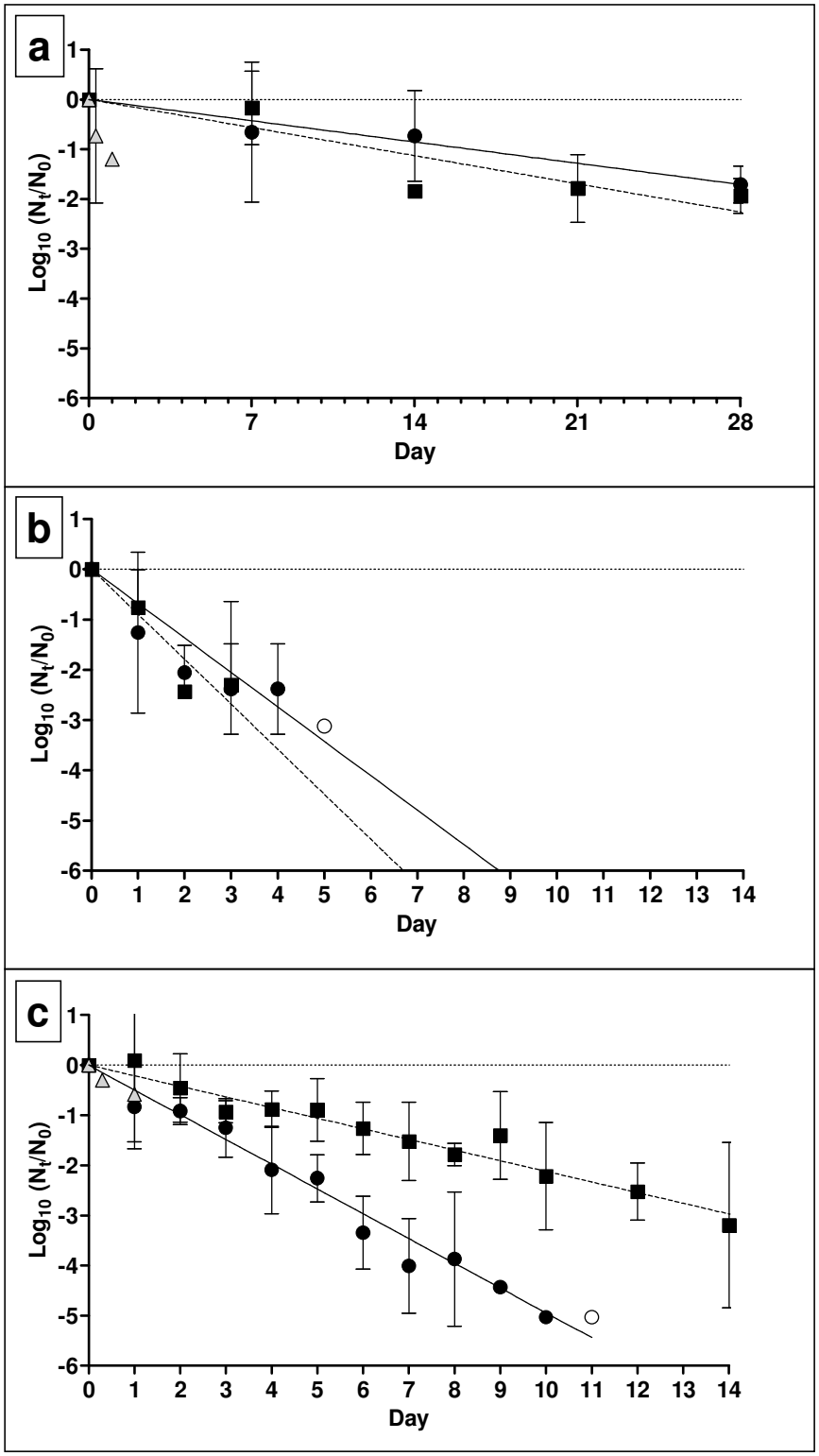

Figure 6-3. Survival of TGEV, MHV, and 229E at 20°C a) 20% RH b) 50% RH c) 80% RH. (Black squares: TGEV; black circles: MHV; gray triangles: 229E; symbols with white centers: sample was below detection limit of the assay; solid line: regression line for TGEV; dashed line: regression line for MHV)

Because of the difficulty of propagating 229E to high titers, it was not possible to observe extensive log reductions of 229E infectivity at the conditions tested However, the infectivity reductions over 24 hours suggest that this virus is inactivated more rapidly than either TGEV or MHV at 20°C. The decline in viral titer at 20°C for TGEV and MHV follows log-linear, first-order kinetics. The slopes of regression lines for TGEV, MHV, and 29E inactivation at 20°C and each RH level are shown in Table 6-2

Table 6-2. Slopes of regression lines for inactivation rates of test coronaviruses at 20°C and 20, 50 and 80% RH

	Slope of regression line (95% CI)		
% RH	TGEV	MHV	229E
20	-0.081 (-0.09 to -0.067)	-0.061 (-0.074 to -0.048)	-1.5 (-2.3 to -0.62)
50	-0.90 (-1.08 to -0.72)	-0.69 (-0.77 to -0.60)	Too few points
80	-0.21 (-0.23 to -0.20)	-0.49 (-0.52 to -0.47)	-0.63 (-0.79 to -0.47)

Comparisons of the slopes of the regression lines for TGEV inactivation rates at 20°C show significant differences among them, with the order of inactivation rates being 50% > 80% > 20%. Inactivates rates of MHV also differed significantly with RH, with the order of inactivation rates again being 50% > 80% > 20%. However, slopes of regression lines at 20% and 80% RH did not differ significantly for 229E, even though rates (slopes) differed by >2-fold, perhaps due to the variability of results. The inactivation rates (slopes) for TGEV and MHV do not differ significantly at 20% and 50% RH, but they are significantly different from the inactivation rate (slope) at 80% RH. 229E appeared to survive better at 80% than 20% RH, but this is based on few data points compared to those for TGEV and MHV.

Figure 6-4 shows virus inactivation rates at 40°C and the same RH levels of 20, 50 and 80%. Overall, viruses were inactivated more rapidly at 40°C than at 20°C. At both

50% and 80% RH, viruses did not survive long enough to dry onto the carrier. Consequently, they were sampled at 2, 4, and 6 hour time points after inoculation, while virus was still wet. At 20% RH, MHV infectivity declined by 4.7 $\log_{10}$ in 5 days, and TGEV infectivity declined by 3.5 $\log_{10}$ in 5 days. Virus infectivity reduction at 50% was more rapid than 80%, with infectivity titer reduction of 4.2 $\log_{10}$ in 18 hours for MHV, and 4.6 $\log_{10}$ in 12 hours for TGEV. Unlike the results at 20°C, the loss of infectivity at 40°Cwas more rapid at 80% RH compared to 50%. At 40°C and 80% RH, MHV, TGEV, and 229E lost 4.1, 2.8, and 1.9 $\log_{10}$ infectious titer at 3 hours, respectively.

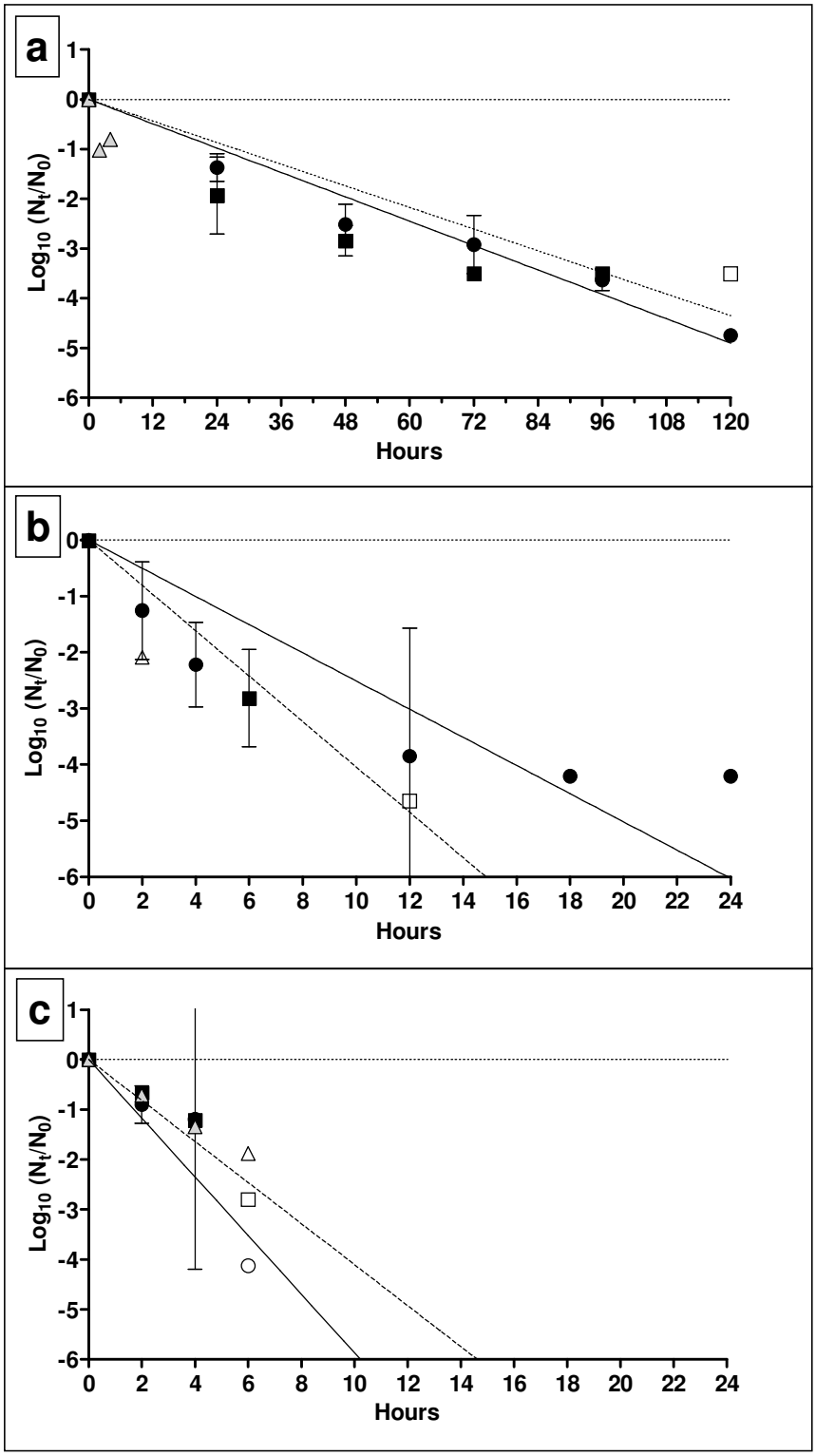

Figure 6-4. Survival of TGEV, MHV, and 229E at 40°C a) 20% RH b) 50% RH c) 80% RH. (Black squares: TGEV; black circles: MHV; gray triangles: 229E; symbols with white centers: sample was below detection limit of the assay; solid line: regression line for TGEV; dashed line: regression line for MHV)

The decline in viral titer at 40°C for TGEV and MHV follows log-linear, first-order kinetics, with one exception. At 40°C and 50% RH, MHV inactivation kinetics appear to display a possible tailing effect, with initial rapid inactivation at 2 and 4 hours a decline in inactivation rate at 12 hours and little further titer decline and stable virus infectivity titers from 12-24 hours. This declining inactivation rate over time or "tailing effect" is not seen with the other viruses tested, and the reasons for it are unclear. The slopes of the fitted regression lines for virus inactivation rates at 40°C and each RH level are shown in Table 6-3.

Table 6-3. Slopes of regression lines for virus inactivation rates at 20°C and RH levels of 20, 50 and 80%

	Slope of regression line (95% CI)		
% RH	TGEV	MHV	229E
20	-0.036 (-0.044 to -0.029	-0.041 (-0.044 to -0.038)	-0.26 (-0.37 to -0.16)
50	-0.40 (-0.44 to -0.37)	-0.25 (-0.31 to -0.19)	too few points
80	-0.41 (-0.47 to -0.36)	-0.59 (-0.70 to -0.47)	-0.32 (-0.36 to -0.29)

The slopes of regression lines for TGEV do not differ significantly between 50% and 80% RH, However, the slope for TGEV inactivation rate at 20% RH is significantly different and much smaller than at 20 and 50% RH. The slopes for MHV inactivation rates at 20°C differ significantly at all three RH levels. For 229E, there is no significant difference between the slopes of inactivation rates at 20% and 80% RH. At 20% RH, rates of inactivation are low and similar for TGEV and MHV, as was seen at 20°C. In contrast, rates of 229E inactivation are more rapid than for the other two viruses. At 50% RH, the inactivation rate is significantly greater for TGEV (-0.40) than for MHV (-0.31), with a rate difference of 0.15 $\log_{10}$ per day. However at 80% RH, the inactivation rates

of TGEV (-0.41) and MHV (-0.59) differ by 0.18 $\log_{10}$/day, but they are not significantly different.

Regression analysis was performed on survival data on SS for each virus, temperature, and RH level. Table 6-4shows predicted values obtained by regression analysis for times to achieve 90%, 99%, and 99.9% reduction of TGEV, MHV, and 229E at each temperature and RH. At 4°C and 20%, viral titer declines so slowly that regression analysis based on this data set (where the longest elapsed time was 28 days) may not be reliable to predict viral reduction over long periods. At ambient temperature (20°C), viruses may be able to survive with no more than 1 $\log_{10}$ or 90% decline in infectivity for 1 or more days on SS surfaces, with the extent of decline depending on the humidity level. For TGEV and MHV, survival at 20°C was longest at 20% RH. Virus survival is lower at higher temperature. At 40°C, time for 1 log10 or 90% reductions occurred in 1-12 hours at 50% and 80% RH, depending on the virus type. At 20% RH and 40°C, virus survival was longer, with 1 $\log_{10}$ or 90% inactivation estimated to occur 3 hours and 1 day, depending on virus type.

Table 6-4. Predicted times (in days) by regression analysis for 90, 99, and 99.9% reduction of infectious virus on a stainless steel surface at varying temperature and RH.

TGEV									
	4°C			20°C			40°C		
reduction	20%	50%	80%	20%	50%	80%	20%	50%	80%
-1 (90%)	50	9	8	12	1	4	1	2 hrs	2 hrs
-2 (99%)	--	18	15	24	2	9	2	4 hrs	4 hrs
-3 (99.9%)	--	28	22	37	3	14	4	7 hrs	7 hrs
MHV									
	4°C			20°C			40°C		
reduction	20%	50%	80%	20%	50%	80%	20%	50%	80%
-1 (90%)	48	7	9	16	1	2	1	3 hrs	1 hr
-2 (99%)	--	13	18	33	3	4	2	8 hrs	3 hrs
-3 (99.9%)	--	20	28	49	4	6	3	12 hrs	5 hrs
229E									
	4°C			20°C			40°C		
reduction	20%	50%	80%	20%	50%	80%	20%	50%	80%
-1 (90%)	50	ND	9	1	ND	1	3 hrs	1 hr	3 hrs
-2 (99%)	--	ND	18	1.5	ND	3	7 hrs	2 hrs	6 hrs
-3 (99.9%)	--	ND	27	2	ND	4	11 hrs	3 hrs	9 hrs

The survival of TGEV and MHV on environmental surfaces was further evaluated using several materials than may serve as fomites in healthcare environments. Virus survival was assessed on polypropylene plastic, laminate, ceramic, latex gloves, nitrile gloves, N95 masks, and contact isolation gowns (made of polypropylene and polyethylene) at constant temperature and RH. These experiments were done at 20°C and 50% RH to simulate ambient temperature and RH conditions in healthcare environments. Experiments were carried out for short periods (2-24 hours) to simulate conditions in healthcare environments, where surfaces are subject to regular cleaning and items such as

gloves and other PPE are worn for short periods during patient care and then discarded. Results are shown in Figure 6-5.

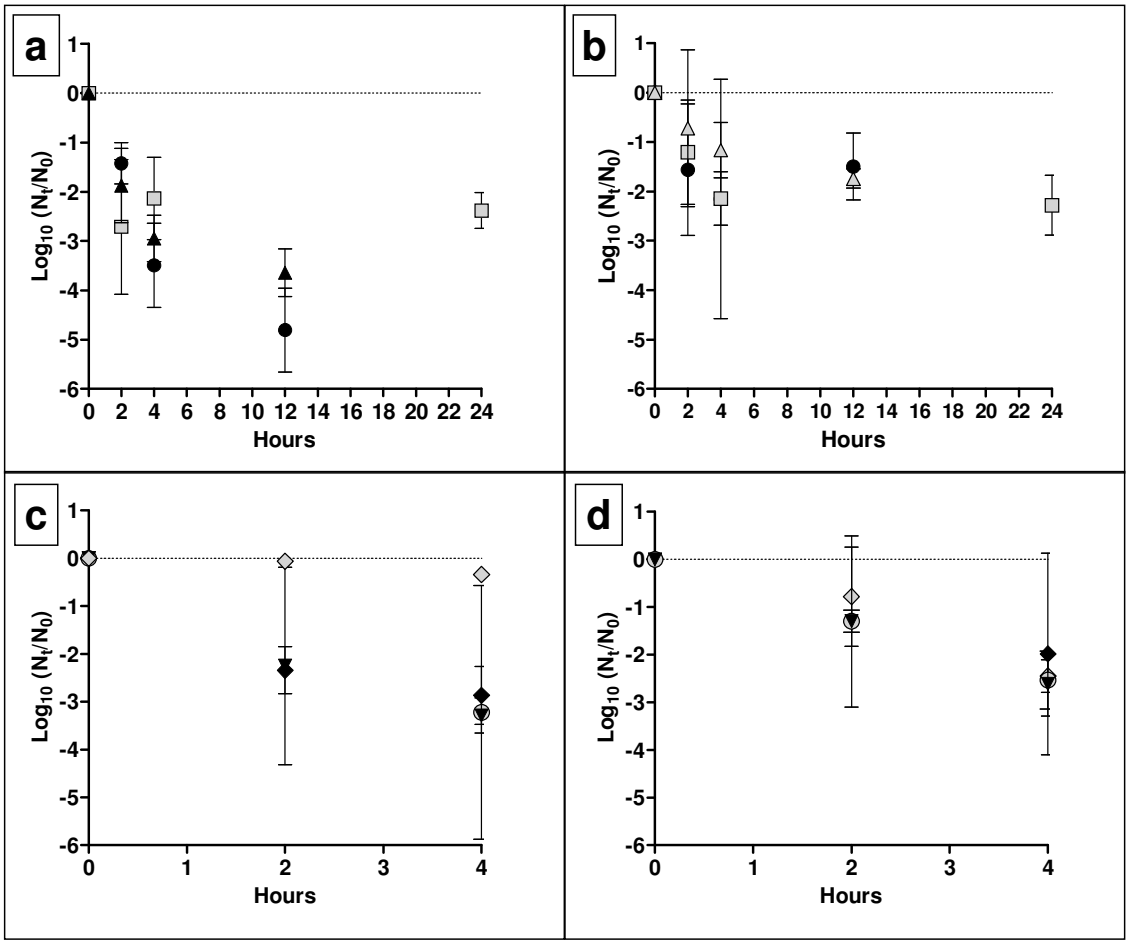

Figure 6-5. a) survival of MHV on nonporous materials b) survival of TGEV on nonporous materials c) survival of MHV on PPE materials d) survival of TGEV on PPE materials (black triangles=ceramic; black circles=plastic; gray squares=laminate; gray diamonds=N95 mask; upside down triangles=latex gloves; black diamonds=gown; gray circles=nitrile glove)

MHV survived for short periods on nonporous plastic, ceramic, and laminate surfaces, with infectivity titer losses of 1.5-2.7 $\log_{10}$ in the first two hours. MHV survived for up to 24 hours on laminate, with approximately 2 $\log_{10}$ infectious titer loss. MHV survival in ceramic and plastic was less than on laminate, with infectivity titer losses of 3.6 $\log_{10}$ and 4.8 $\log_{10}$ for respectively at 12 hours (Figure 1a). On PPE, virus survival varied by

material type. MHV infectivity was stable on N95 respirator material for up to 4 hours. On contact isolation gowns and latex and nitrile gloves, MHV infectivity titer declined ~3 $\log_{10}$ within 4 hours. MHV survival results on laminate were similar to those for TGEV, with 2 $\log_{10}$ loss of infectivity over 24 hours. TGEV survived less well than MHV on N95 respirator material, contact isolation gowns, N95 respirators, nitrile gloves, and latex gloves, with infectivity titer declines of 2-2.6 $\log_{10}$ within 4 hours.

Discussion

These results of these studies show that if deposited in high numbers (10^3-10^4 $\log_{10}$), coronaviruses dried onto surfaces may survive for days at temperatures and humidity levels found in health care environments. Overall, virus survival was enhanced by lower temperatures, with progressively slower inactivation rates at 4°C>20°C>40°C. Similar relationships have been observed for TGEV and MHV survival in water (Casanova *et al.*, unpublished data), canine coronaviruses in liquid suspension (Tennant, Gaskell, and Gaskell, 1994), and coronaviruses in aerosols (Ijaz *et al.*, 1985). In general virus survival is longer at lower (4-6°C) compared to ambient (20°C) temperatures. The results of this study are also consistent with those obtained for other viruses on surfaces. Survival of non-enveloped viruses such as hepatitis A, poliovirus and adenovirus is also greater at low temperatures (Abad, Pinto, and Bosch, 1994; Abad *et al.*, 2001; Mbithi, Springthorpe, and Sattar, 1991; Sattar *et al.*, 1986). The effect of temperature on virus survival on surfaces appears to be similar to the effects on virus survival in other environmental media such as air, water and soil.

In these experiments, the relationship between virus inactivation on surfaces and relative humidity level was not monotonic, which is consistent with survival observations

made by other investigators for some non-enveloped virus dried on surfaces. As was seen with TGEV and MHV, survival of some non-enveloped viruses is greater at high than at low relative humidity (Abad, Pinto, and Bosch, 1994; Sattar *et al*., 1988). This has been observed for human rotavirus on stainless steel, plastic, and glass, on which rates of viral inactivation were higher at 50% RH than at 25%, and higher at 80% RH than at 50% (Sattar *et al.*, 1986). Human rhinovirus survival had an opposite relationship with RH, with survival on surfaces of 14 hours at high RH but only 2 hr at low RH (Sattar *et al.*, 1987). Studies of hepatitis A survival on surfaces between 25 and 95% RH, report survival is inversely proportional to the RH level

At 20°C, inactivation of TGEV and MHV in these experiments was slower at 20% RH compared to 50% RH and 80% RH. Inactivation was sometimes also slower at 80% RH than 50%, but the difference was not always significant. Previous studies of TGEV in aerosols have found greater viral recovery from aerosols at low RH than at high RH (Kim *et al*., 2007), suggesting possible longer survival of coronaviruses at low RH. This is consistent with experiments examining the survival of human coronavirus 229E in aerosols, which found that aerosolize virus survived longer at low RH compared to high RH (20% inactivation after 25 hours at 20% RH vs. 90% inactivation after 25 hours at 80% RH). The relationship between relative humidity and virus survival in aerosols was not monotonic; the trend from greatest to least inactivation was 80%>20%>50% (Ijaz *et al*., 1985). Results from this study suggest a roughly U-shaped relationship (or inverted triangular distribution) on surfaces, with greater survival or a protective effect at moderate (50%) RH and lower survival at both high (80%) and low RH (20%) RH. Conflicting results in previous literature suggest that this relationship may vary greatly by

virus type. Because much of the previous work done has been with non-enveloped viruses, the results may not be directly applicable to predicting the survival of enveloped viruses on surfaces.

Other investigators have also found that when virus survival is compared at specific RH levels but different temperatures, the basic relationship between survival and RH level remained the same. However, the effect of RH level on virus survival was "less pronounced" at lower temperatures compared to higher temperatures (Mbithi, Springthorpe, and Sattar, 1991). This effect was also observed with coronaviruses. Inactivation of TGEV, MHV and 229E was slower at 4°C than at 20°C, but at both temperatures, viral inactivation was slower at 20% RH than at 50% or 80%. Virus inactivation was also slower at 80% RH than at 50%. At 40°C, the same protective effect of low RH was seen at 20% RH compared to 50% and 80%, but overall, inactivation was more rapid at all three RH levels at this temperature. It may be that at 40°C, temperature effects predominate in causing viral inactivation, with RH levels playing a lesser role than they do at lower temperatures. The coronavirus data presented here, as well as previous findings with non-enveloped viruses, suggest that although rates of viral inactivation are slower at lower temperatures, there are still differential effects of humidity on viral survival at varying temperatures. Because the relationship between temperature, humidity and virus inactivation is still not entirely clear, and may differ by virus type (Abad, Pinto, and Bosch, 1994; Mbithi, Springthorpe, and Sattar, 1991), further comparative studies are recommended.

In this study, human coronavirus 229E infectivity declined by 1.2 $\log_{10}$ after 24 hours at 20% RH and by 0.6 $\log_{10}$ after 24 hours at 80% RH. Previous studies of 229E survival

found that it lost ~3 $\log_{10}$ of infectivity over 6 hours on an aluminum surface at 20°C when RH was between 50 and 75% (Sizun, Yu, and Talbot, 2000). In our studies, 229E did not survive drying onto stainless steel carriers at 20°C and 50% RH, a process that took 4-6 hours at this temperature and RH. The rapid inactivation of 229E observed by Sizun *et al*. may represent the effect of moderate (~50%) RH on virus survival. The results of this study also suggest that TGEV and MHV dried onto surfaces at ambient temperatures are much more resistant to inactivation than 229E. It is difficult to determine from existing data whether TGEV and MHV are more or less resistant to inactivation on surfaces than SARS CoV. SARS-CoV has been reported to survive for 36 hours on stainless steel when 10^4 $TCID_{50}$ are deposited initially (WHO, 2003), but the temperature and humidity conditions for this experiment were not reported, making comparisons difficult. However, a reduction of 4 $\log_{10}$ infectious titer in 36 hours was greater than that seen for either TGEV or MHV at 20°C at any RH level in this study. Rabenau *et al*. (2005) reported much slower inactivation of SARS-CoV on a polystyrene surface, with a reduction of 4 $\log_{10}$ after 9 days (RH conditions not reported). This observed persistence of SAR-CoV is consistent with observations of TGEV and MHV persistence at 20°C and 50% RH in this study. Based on their observed comparable persistence, TGEV and MHV may be suitable models for survival and inactivation of SARS-CoV on surfaces Coronavirus 229E appears to be inactivated much more quickly than SARS-CoV and therefore may be less effective as a surrogate for it in this context. However, more data are needed on the survival rates and inactivation kinetics of SARS-CoV itself before these relationships with other coronaviruses can be definitively established.

These results of this study also show that TGEV and MHV deposited in high numbers (10^4-10^5 MPN) survive on materials used to make PPE, although inactivation is more rapid than on stainless steel surfaces. This finding differs from previous studies of survival of human coronavirus 229E on latex surfaces, in which survival on latex and stainless steel was similar (Sizun, Yu, and Talbot, 2000). TGEV and MHV infectivity on latex at 20°C and 50% RH in this declined by ~3 $\log_{10}$ in 4 hours. This infectivity decline is similar to 229E, which was reported to decline by ~3 $\log_{10}$ in 6 hours at 20°C when RH was between 50 and 75% (Sizun, Yu, and Talbot, 2000). Because humidity was not controlled in the previous studies of 229E, it is difficult to determine what the contributions of RH conditions and surface type to virus stability or inactivation in those studies. The data for TGEV and MHV suggest that contact isolation gowns, N95 respirators, and gloves contribute to the loss of virus infectivity on these materials. Consistent with results for non-enveloped viruses on stainless steel surfaces, coronavirus infectivity also appears to be less persistent on latex than are non-enveloped viruses. Non-enveloped viruses persist for weeks on latex with only 1-3 $\log_{10}$ decline in infectivity (Abad, Pinto, and Bosch, 1994). In the survival experiments conducted on PPE, ceramic, laminate, and plastic at 50 and 80% RH, the viral inoculum is drying in the first 4-5 hours at these RH levels. The shape of the inactivation curve within the first several hours at these RH levels suggests that inactivation during drying may not follow first-order kinetics. Instead of linear kinetics throughout the duration of the experiment, it may be that viral inactivation during drying follows non-first-order kinetics, and inactivation after drying is complete then follows first-order kinetics. However, further experiments

are needed to more precisely define the kinetics of viral inactivation during the drying process.

The mechanisms of inactivation of viruses on surfaces have not been completely elucidated, but a number of contributing factors may have a role . Studies have shown that virus inactivation can take place at the air-water interface (AWI) of a solution (Thompson and Yates, 1999; Trouwborst *et al*., 1974). The surfaces of viral capsids can have both hydrophobic and hydrophilic regions, which may drive them to accumulate at the AWI as hydrophobic regions of the virions partition out of solution and hydrophilic regions remain in solution (Thompson *et al*., 1998). It is possible that forces exerted on viral particles as they partition at the AWI lead to structural damage and viral inactivation (Trouwborst *et al*., 1974). This phenomenon may play a role in inactivation of viruses in liquid suspensions that are placed on surfaces, because droplets of liquid will have an AWI on the surface of the droplet until desiccation has taken place. Interactions between the virus and the AWI may also help to explain why enveloped viruses on surfaces do not survive as long as non-enveloped viruses. Viruses with hydrophobic surface properties have a higher affinity for the AWI (Thompson and Yates, 1999), resulting in inactivation by protein unfolding and damage (Trouwborst *et al*., 1974). The lipid envelopes on the surfaces of coronaviruses may have this affinity for the AWI, resulting in greater inactivation at the interface compared to non-enveloped viruses. Coronaviruses also have a spike protein which protrudes from the viral surface. This protein has both hydrophobic and polar regions (Bosch *et al*., 2003; Spiga *et al*., 2003), which may cause viral particles to partition along the AWI, leading to viral inactivation.

On surfaces, the AWI may not be the only site where viral inactivation is taking place. Studies of bacteriophage survival at the AWI in contact with surfaces have identified a role for the location where the liquid, solid, and gas phases meet, called the triple-phase-boundary (TPB). Forces operate at the boundary where liquid, air, and a hydrophobic surface meet, such as reorientation of water molecules as they encounter the hydrophobic surface. Viruses that partition at the TPB can be structurally damaged by these forces, leading to inactivation (Thompson *et al*., 1998). This may explain virus inactivation on surfaces such as contact isolation gowns, which can be made of hydrophobic materials such as polyethylene. This partitioning at the AWI may also explain the differential susceptibility of individual members of the *Coronaviridae* to inactivation on surfaces. The major coronavirus surface protein is the spike protein, which plays a vital role in viral entry into the cell (Bosch *et al*., 2003). Comparative studies of the spike protein structure of coronaviruses have found differences in the amino acid sequence in portions of the spike proteins of MHV and 229E (Bosch *et al*., 2003). These amino acid sequence variations may lead to structural variations that cause different coronaviruses to partition differently at the AWI, causing some coronaviruses to be inactivated more quickly or to a greater extent by forces at the interface than others. Previous investigators have found that the effect of humidity on viral survival on surfaces can differ for structurally similar viruses belonging to the same family (Mbithi, Springthorpe, and Sattar, 1991), and these small structural variations in important viral proteins may partially explain why the relationship between RH and survival on surfaces differs even among closely related viruses.

Other investigators have suggested that viral inactivation during the desiccation step is an important determinant of survival of viruses on surfaces (Abad, Pinto, and Bosch, 1994). During desiccation, the loss of water molecules from a viral suspension on a surface triggers several processes that lead to viral inactivation. Lipid membranes undergo phase changes as water molecules are lost, destroying their native structures. As lipid membranes undergo phase changes, the proteins associated with these membranes may also undergo changes. Protein groups brought into contact by changes in the shape of the lipid membrane may undergo cross-linking reactions and changes in tertiary protein structures. Proteins and nucleic acids can crosslink, both with other like molecules and with each other (Cox, 1992). These changes in lipid and protein structures could be the mechanism by which enveloped viruses are inactivated as they become desiccated on surfaces. During desiccation, amino acids in viral proteins can also undergo Maillard reactions as water molecules are lost, resulting in structural changes. Lipids that are oxidized during the desiccation process also form peroxides and hydroperoxides, which can also participate in Maillard reactions, driving further protein damage (Cox, 1992).

As discussed above, mechanisms of virus inactivation on surfaces may involve both desiccation and interaction at the AWI, with the contribution of each depending on the RH level. At low RH, desiccation is relatively rapid, and viruses may have little time to diffuse toward the AWI and undergo inactivation there. At low RH, oxidation and Maillard reactions occurring during desiccation may be the predominant mechanisms of inactivation, with little opportunity for inactivation at the AWI because water is lost quickly (complete drying at 20% RH in these studies was observed in ~3 hours). If

relative humidity levels are high (80%), the rate of loss of water molecules may be very slow (drying times in these studies at 80% RH were >6 hours). During the time the virus remains in liquid suspension, the main mechanism may be inactivation taking place at the AWI, rather than inactivation by desiccation effects The altter mechanism may predominate later when most of the liquid has evaporated away. In addition, at high RH, the hydrophobicity of the AWI is decreased (Mbithi, Springthorpe, and Sattar, 1991). This may have protective effects, possibly decreasing the partitioning of viruses at the AWI and slowing viral inactivation. However, when RH is around 50%, where liquid suspension evaporates slowly over a period of hours (4-5 in these studies), then these two mechanisms of inactivation- inactivation at the AWI and inactivation by desiccation- may be operating simultaneously. At moderate RH, lower numbers of water molecules in the air may increase the hydrophobicity of the AWI compared to high RH, increasing viral partitioning at the interface and resulting in increasing rates of viral inactivation. Because the AWI persists for a long period during slow desiccation, viruses in the suspension have more opportunities to diffuse toward this more hydrophobic AWI. Also, as water molecules are lost during desiccation, two processes can be taking place. First, there is the lipid oxidation and Maillard reactions from desiccation itself, with maximum rates of Maillard reactions occur when RH levels are 50-80% (Cox, 1992). Second, more viruses in various layers of the viral suspension may be exposed to the AWI as water molecules are progressively lost from the droplet of virus suspension. Both of these processes last longer than they would at low RH, possibly resulting in greater viral inactivation. This may serve as a partial explanation for why viral inactivation appears to be more rapid at 50% RH than at 20% or 80%.

There may also be mechanisms of inactivation that are specific to the structural features of the coronaviruses. The coronavirus spike protein, the main viral surface protein, contains a cysteine rich domain in the transmembrane anchor, which is necessary for viral fusion with cells (Chang *et al*., 2000). Cysteine residues contain sulfur groups, which form disulfide bridges when oxidized. Disulfide bridge formation irreversibly alters the structure of the protein. It may be that oxidation caused by free radicals resulting from the breakdown of the lipid viral envelope (Cox, 1992) results in the formation of disulfide bridges in the transmembrane domain, irreversibly altering a section of the protein necessary for viral fusion with host cells.

The results of this study show that coronaviruses can survive on surfaces anywhere from hours to weeks, depending on the temperature and relative humidity levels. At ambient temperatures around 20°C, these viruses can survive for 2 days while losing only 1-2 $\log_{10}$ infectivity, depending on the humidity level. Therefore, if deposited in high numbers, they can potentially survive on surfaces in healthcare environments for days. If these viruses contaminate items of PPE in high numbers, they may survive for the length of time that PPE is worn (typically <1hr) in numbers high enough to pose a risk of viral transmission if people come in contact with contaminated PPE. This could pose a serious human health risk should SARS-CoV ever re-emerge. Evidence of SARS-CoV contamination of hospital surfaces was been found during the SARS outbreak. Sampling of surfaces in hospitals during the outbreak, including surfaces in patient rooms and areas on floors used for isolation of SARS patients, found SARS-CoV nucleic acids on surfaces and inanimate objects in patient rooms, nurses' stations, and public areas of the hospital (Booth *et al.*, 2005; Dowell *et al*, 2004). Infectious virus was not cultured from any of the

samples, so it is unclear whether the presence of viral nucleic acids indicates that infectious SARS-CoV is present on hospital surfaces in outbreak settings. The presence of nucleic acids, however, suggests the possibility that infectious virus was present on these surfaces for a period of time, and these surfaces could become sources of further viral transmission. If these surfaces become contaminated by body fluids from SARS patients during illness, these fluids could potentially deposit SARS-CoV in high numbers. There have been several studies quantifying the viral load in body fluids from SARS patients. Wong *et al.* (2005) examined nasopharyngeal swabs of SARS patients and found that viral load ranged from 10^4 to 10^7 RNA copies/mL; Cheng *et al.* (2004) found that viral load in nasopharyngeal aspirates peaked around day 12-14 of infection, at 10^5 copies/mL. Their findings are similar to those of Hung *et al.* (2004), who found that mean viral load in nasopharyngeal aspirates around day 10–15 of infection was 10^5 copies/mL. Peiris *et al.* (2003) found that peak viral load in nasopharyngeal aspirates around day 10 of infection ranged from 10^6 to 10^8 copies/mL. Together, these findings suggest that respiratory secretions from SARS patients may contain high numbers of viruses, which could potentially result in deposition of high numbers of viruses on hospital surfaces or healthcare workers' PPE. If this happens, these viruses could remain infectious on surfaces long enough for other people to come in contact with them, posing a risk for disease transmission. The results of this study suggest that TGEV and MHV could serve as conservative surrogates for modeling the risk of SARS-CoV presence and persistence on environmental surfaces to pose a risk of exposure and transmission via healthcare surfaces and PPE items. These viruses may be useful models in studies to determine ways to interrupt this route of SARS-CoV disease transmission.

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CHAPTER 7

Summary and Conclusions

The results of these studies demonstrate that members of the *Coronaviridae* can survive and remain infectious for long periods of time in water, on surfaces, and on fomites, including personal protective equipment designed to interrupt the transmission of viruses to healthcare workers. These environmental media play roles in several potential pathways for virus transmission. These include:

- Inhalation of droplets and aerosols from virus-contaminated water
- Deposition of viruses on surfaces when virus-containing droplets and aerosols settle out of the air or are removed other loss mechanisms (impaction, electrostatic deposition, etc.)
- Acquisition of infectious virus from direct contact with virus-contaminated surfaces
- Acquisition of infectious viruses from PPE when PPE items are handled during removal

Methods for virus recovery and the data on virus survival from these studies can be used to more accurately model and assess the risks of coronavirus transmission via surfaces and fomites in healthcare environments.

Examination of the kinetics of coronavirus survival in water provides some insights into the persistence of coronaviruses in different environmental media. These studies demonstrated that TGEV and MHV can survive and remain infectious for long periods in different water types ranging from highly treated water, to surface water to sewage at both low (4^{o}C) and at typical temperate ambient (25°C) temperatures. In all water types,

the titer of infectious virus declined more rapidly at 25°C than at 4°C. At 4°C, infectious virus titers declined only slowly and either by low to moderate levels (up to 2 $\log_{10}$ at 14 days) or not at all. In reagent-grade water at 25°C, infectious virus titers declined more rapidly than at 4°C, with the extent of reduction differing with water quality. At 25°C the infectivity of TGEV in reagent grade water declined by approximately 0.6 $\log_{10}$ per week, vs. 1.5 $\log_{10}$ per week in pasteurized settled sewage. MHV in reagent grade water at 25°C declined by approximately 0.8 $\log_{10}$ per week, vs. 2 $\log_{10}$ per week in pasteurized settled sewage. Temperature and incubation time were significant predictors of viral reduction, which is consistent with previous studies of viral survival in water (Yates, Gerba, and Kelley, 1985; Enriquez, Hurst, and Gerba, 1995). Water type was a significant predictor of the rate of viral reduction, with greater reduction seen in pasteurized settled sewage as compared to reagent grade water. There are several factors that could contribute to greater reduction in more contaminated water, including high molecular weight dissolved matter (Noble, and Fuhrman, 1997), particulate matter (possibly due to irreversible viral binding to particles) (Suttle and Chen, 1992), chemical constituents with antiviral activity, and bacteria which feed on the viral constituents such as proteins, producing metabolites that are toxic to viruses, and releasing proteolytic enzymes that damage virus structures (Deng and Cliver, 1992; Deng and Cliver, 1995; Herrmann *et al*., 1974).

Long-term coronavirus survival in water has implications for human health. It has been established with other human pathogens that formation of droplets and aerosols from water contaminated with microorganisms can serve as a vehicle for transmission of both respiratory and enteric infections. The unique Amoy Gardens SARS outbreak, where a combination of factors resulted in aqueous droplets of fecally shed virus being

drawn from the plumbing system into living spaces and inhaled by building occupants, demonstrated that water aerosols have the potential to spread coronavirus infection (McKinney *et al*., 2006; WHO, 2003). This airborne route of exposure to waterborne pathogens has been recognized with other infectious diseases. Epidemiologic studies have found heightened disease risks among people in communities exposed to sprinkler irrigation using wastewater (Fattal *et al*., 1986), including risks of clinical influenza and infectious hepatitis (Katzenelson, Buium, and Shuval, 1976). However, some of the evidence on the infectious disease risks from this roure of transmission is conflicting. Other studies have found no differences in disease rates from exposure to spray-irrigated wastewater (Shuval, Yekutiel, and Fattal, 1989).

The available evidence suggests that a potential human health risk from exposure to water droplets and aerosols contaminated with viruses, including coronaviruses, exists, but the extent of this risk is unclear. It may be possible that if SARS-CoV-contaminated water or sewage becomes aerosolized, it could potentially transmit virus to large groups of people, even those who have never come in contact with an infected individual. The persistence of coronaviruses in water and sewage suggests that quarantine measures, which proved effective in containing the last SARS outbreak, could be undermined if the risk of contaminated water is not considered. However, the magnitude of that risk is still unclear, and survival in water is only one piece of evidence required to assess this category of risk. The risks should be further explored using data on virus survival in droplets and aerosols, exposure data linked to measured health effects from epidemiological studies and risk assessment modeling. Data on the survival of coronaviruses in water as well as in airborne droplets and aerosols can be used in these

risk assessment models to more accurately quantify risks posed by the water droplet or aerosol pathways and design strategies to control them.

The risk posed by viruses in airborne droplet and aerosols is not limited to inhalation of these droplets or aerosols. Coronaviruses can survive for hours in aerosolized states (Ijaz *et al*., 1985), and the fate of these aerosols in the environment has implications for infection control. As droplets and aerosols settle, they can land on environmental surfaces. The deposition of these particles on surfaces could pose a risk of infection to people who come in contact with contaminated surfaces. Studies have shown that people transfer viruses from surface to hands and hands to surfaces when touching contaminated surfaces, and that these surfaces can transmit infection (Ansari *et al.*, 1991; Ansari *et al.*, 1998; Gwaltney and Hendley, 1982; Mbithi *et al.*, 1992; Rusin, Maxwell, and Gerba, 2002; Sattar *et al.*, 1993).

The results of these studies show that members of the coronavirus family have the potential to remain infectious for hours to days when dried onto environmental surfaces, with important implications for these surface-to-hand and hand-to-surface transmission pathways. The magnitude of the risk from these transmission pathways is influenced by environmental conditions which affect viral survival. As found in water, temperature is an important factor in virus survival; low temperatures reduce the rates of viral inactivation on surfaces just as they do in water. The rate of viral inactivation is also dependent on the relative humidity level of the environment, with high (80%) and low (20%) RH being more favorable to virus survival than moderate (50%) RH. If deposited in high numbers, coronaviruses dried onto surfaces may survive for days at temperatures and humidity levels found in healthcare environments. In these experiments, the

relationship between virus inactivation on surfaces and relative humidity level was not straightforward, which is consistent with observations made by other investigators of survival kinetics of other viruses dried on surfaces. Results from this study suggest a roughly U-shaped relationship in which, compared to moderate (50%) RH, high RH has a protective effect for viruses dried onto surfaces, but lower humidity levels may also have this effect. Conflicting results in previous literature suggest the relationship between humidity and virus inactivation is still not entirely clear, and may differ by virus type (Abad, Pinto, and Bosch, 1994; Mbithi, Springthorpe, and Sattar, 1991). Much of the previous work done has been with non-enveloped viruses, and the results may not be directly applicable to predicting the survival of enveloped viruses on surfaces. The coronavirus data presented here, as well as previous findings with non-enveloped viruses, suggest that rates of viral inactivation are slower at lower temperatures, but effects of relative humidity on viral survival at varying temperatures are differential, with often greater survival at RH extremes than at 50% RH.

The data from this study offer some insight into the comparative survival of different coronaviruses on surfaces. TGEV and MHV dried onto surfaces at ambient temperatures appear to be much more resistant to inactivation than is human coronavirus 229E. It is difficult to determine from existing data whether TGEV and MHV are more or less resistant to inactivation on surfaces than SARS CoV. This is because there are currently only limited data on the survival of SARS-CoV on surfaces. Rabenau *et al*. (2005) reported SARS-CoV exhibited a reduction of 4 $\log_{10}$ after 9 days on a polystyrene surface. In that study it was not reported whether temperature and humidity conditions were controlled and at what levels. This extent of SARS-CoV inactivation is consistent with

observations of inactivation rates for TGEV and MHV at 20°C and 50% RH in this study. This consistency in virus inactivation rates suggests that TGEV and MHV may be suitable models for survival and inactivation of SARS-CoV on surfaces. In contrast, 229E appears to be inactivated much more quickly than SARS-CoV and therefore is inadequate to model SARS-CoV survival. However, more data are needed on the inactivation rates of SARS-CoV itself before these relationships can be definitively established.

The mechanisms of inactivation of viruses on surfaces have not been completely elucidated, but several possible mechanisms exist. Some are specific to the structure of coronaviruses, and some are generally applicable to all virus types. They include:

- Inactivation at the air-water interface (AWI) of a solution, due to hydrophobic regions of the virus structure partitioning out of solution and hydrophilic regions remain in solution (Thompson *et al*., 1998)
- Affinity of the lipid envelopes on the surfaces of coronaviruses for the AWI, resulting in greater inactivation at the interface compared to non-enveloped viruses
- Partitioning of the coronavirus spike protein, which has both hydrophobic and polar regions (Bosch *et al*., 2003l; Spiga *et al*., 2003), at the AWI, leading to viral inactivation
- Inactivation by viruses partitioning at the triple-phase-boundary (TPB) where liquid, air, and a hydrophobic surface meet
- Amino acid sequence variations in portions of the spike proteins of individual members of the *Coronaviridae*, causing different species of coronaviruses to partition differently at the AWI, and causing some coronaviruses to be inactivated more quickly than others by forces at the interface
- Loss of water molecules from a viral suspension on a surface during desiccation
- Phase changes in lipid membranes during desiccation, destroying their native structures

- Cross-linking reactions and changes in tertiary protein structures for protein groups brought into contact by changes in the shape of the lipid membrane during desiccation
- Viral proteins undergoing Maillard reactions as water molecules are lost, resulting in structural changes
- Oxidation of lipids during the desiccation process to form peroxides and hydroperoxides, which can also participate in Maillard reactions, driving further protein damage (Cox, 1992)
- Desiccation-induced oxidation resulting in the formation of disulfide bridges in the transmembrane domain of the coronavirus spike protein, irreversibly altering the structure of the protein

On surfaces, mechanisms of virus inactivation may involve both desiccation and interaction at the AWI, with the contribution of each depending on the RH level. These data show that coronaviruses can survive on surfaces anywhere from hours to weeks, depending on the temperature and relative humidity levels. Times required for 99% inactivation ranged from 3-5 days at 20°C and high humidity, to weeks at 4°C and low humidity. If deposited in high numbers, infectious coronaviruses and other viruses can potentially survive for days on surfaces in healthcare environments, such as tables, bedrails, doorknobs, faucets, desks, phones, countertops, computer keyboards and mouses, and medical equipment (Boone and Gerba, 2007). As with contaminated aerosols, contaminated surfaces could continue to serve as sources of transmission for viruses such as SARS-CoV after infected individuals are no longer present in the contaminated environment.

The survival of coronaviruses on surfaces also has important implications for transmission via contaminated fomites in healthcare environments, particularly items of personal protective equipment (PPE) used by HCWs. These studies show that TGEV and

MHV deposited in high numbers (10^4-10^5 MPN) survive on different non-porous and porous materials of healthcare personal protective equipment. Studies quantifying the viral load in body fluids from SARS patients estimate viral load in respiratory secretions ranging from 10^4 to 10^8 units (gene copies)/mL depending on the stage of infection. If secretions containing these levels of viral load contaminate items of PPE, some viruses may survive for the length of time that PPE is worn during patient care activities (typically <1 hr) in numbers high enough to pose a risk of viral transmission when PPE is handled during removal. This could pose a serious human health risk should SARS-CoV ever re-emerge.

There are still knowledge gaps in assessing the risk of viral disease transmission posed by handling contaminated PPE. Accurate measurement of levels of viral contamination and the length of viral survival on PPE items is vital to accurately asses these risks, and studies of viral loads and survival on PPE items require quantitative and efficient methods for recovering viruses from PPE. To date, there have been few studies assessing the survival of pathogens on materials used to make personal protective equipment (Yassi *et al*., 2005). The existing literature on viral survival on PPE encompasses only some materials, and studies use a variety of methods to recover viruses from test materials, making comparisons between studies difficult. Different methods for viral recovery may have different recovery efficiencies, especially if not specifically tailored for the recovery of viruses.

This study demonstrated that a protein-based eluent, beef extract, is effective for eluting viruses from a range of PPE materials, possibly due to the disruption of both charged and hydrophobic interactions between the virus and the PPE surface. Beef

extract may also be efficacious for the elution of other types of viruses from PPE, including enveloped viruses, but further research in this area is needed. The recovery methods developed in these studies using a model virus (MS2) can be used to expand our knowledge of how PPE becomes contaminated by viruses, how long these viruses survive on contaminated PPE, and the extent to which these viruses can be transferred to other people and pose health risks.

These studies of TGEV and MHV on PPE materials suggest that coronaviruses have the potential to survive on such materials for periods longer than single-use PPE is usually worn, creating the potential for viral transfer when PPE is handled after wearing. Removing PPE after patient care without contaminating skin or clothes is important for reducing HCWs' infection risk. If patients shed viruses onto healthcare workers' PPE in the course of patient care, these viruses can remain infectious when PPE is removed. Transfer of viruses from experimentally contaminated fabrics (Rusin, Maxwell, and Gerba, 2002), plastic surfaces (Gwaltney and Hendley, 1982), and gloves (Hall, Douglas, and Geiman, 1980) to hands has been demonstrated, suggesting that viruses can transfer from PPE to hands when contaminated items are handled in the course of removal and disposal. In addition, contamination can be present on skin after exposure to pathogens even when PPE is worn (Zamora *et al*., 2006), and may be transferred to used items of PPE if they are handled after removal. Virus transfer between hands and PPE items can encourage both accidental autoinoculation by the healthcare worker and subsequent transmission of viruses to other patients, staff, or family members, especially when inadequate hand hygiene is practiced (Pittet *et al*., 2006).

Given that PPE may be contaminated with infectious virus when it is removed after patient care, developing and validating an algorithm and protocol for the removal of PPE that prevents contamination of the skin and clothes of HCWs is key to interrupting nosocomial transmission of potentially serious infectious diseases, including SARS, avian influenza, viral hemorrhagic fevers and poxvirus diseases (e.g., monkey pox). The human volunteer experiments carried out using MS2 and the current CDC algorithm and protocol for PPE removal demonstrate viral transfer from PPE to skin and clothes during PPE removal. These results demonstrate that the current CDC algorithm/protocol is insufficient to protect HCWs from contamination during PPE removal. However, several potential options exist to that might prevent or further reduce such contamination, including double gloving, use of surgical protocols for PPE removal, and use of PPE impregnated with antimicrobial agents.

The first two options ensure that PPE is not handled with ungloved hands. A double glove removal sequence would begin with removal of the outer glove, followed by goggles or face shield, gown, and respirator/mask, and finishing with removal of the inner glove followed by hand hygiene. Using this method, handling of PPE with ungloved hands is avoided. The use of an inner glove ½ size larger than usually worn may be used to improve dexterity and reduce constriction when double gloving. Borrowing PPE protocols from surgery, where the ends of gown sleeves are tucked underneath gloves during wear, might also reduce contamination. When finished, goggles and respirator are removed first, and gown and gloves are then removed together by peeling off both at the same time, again avoiding handling PPE with ungloved hands. Use of PPE impregnated with antimicrobial agents may be able to reduce contamination

without the need for behavior change. Further research is needed to determine what antiviral agents would be effective when incorporated into or applied to PPE without posing unacceptable toxicity risks to users or their contacts.

This study also indicates the need for continued emphasis on hand hygiene. A barrier to improving hand hygiene compliance rates is the belief that gloves make hand hygiene unnecessary (Pittet *et al*., 2001). This belief is contradicted by our study and others showing that organisms can spread from gloves to hands after glove removal (Doebbeling *et al*., 1988). Even if double gloving is incorporated into protocols for PPE use, it is vital to emphasize that this procedure is not a substitute for proper hand hygiene. Before these or other candidate methods are introduced into clinical practice, their impact on the safety of HCWs should be validated by testing using methods such as those developed and applied in these studies.

Conclusions

- TGEV and MHV can survive and remain infectious for long periods in different water types including highly treated water, surface water, and sewage
- TGEV and MHV can survive and remain infectious at both low (4°C) and at typical temperate ambient (25°C) temperatures. Estimated times for 99% infectious virus reduction (1% initial virus survival) ranged from 49 to >365 days at 4°C and from 7 to 22 days at 25°C, depending on water quality
- In all water types, the titer of infectious virus declined more rapidly at 25°C than at 4°C.
- Water type, incubation time, and temperature were significant predictors of $\log_{10}$ viral reduction kinetics.
- TGEV and MHV may serve as conservative indicators of the survival of SARS-CoV in water and sewage.
- The observed survival of coronaviruses at low and moderate temperatures in highly treated potable waters has important exposure risk implications for its spread via fecally contaminated water as droplets or aerosols, should it re-emerge in human populations.
- A protein-based eluent, beef extract, is effective for efficiently eluting viruses from a range of PPE materials, possibly due to the disruption of both charged and hydrophobic interactions between the virus and the surface.
- Elution with 1.5% beef extract pH 7.5 followed by two-step enrichment assay can efficiently recover infectious MS2 from contaminated PPE that has been worn during the performance of a healthcare task
- The methods developed for elution and assay of infectious MS2 from PPE can be used to conduct further quantitative and rigorous studies of viral survival on PPE using model viruses, in order to produce data that can be used for risk assessments in infection control and healthcare worker protection.
- The results of this study document viral transfer from PPE to other PPE sites and to skin.

- Such virus transfer demonstrates that the current CDC algorithm and protocol is insufficient to protect HCWs from contamination during PPE removal
- Developing and validating an algorithm or protocol for the removal of PPE that prevents contamination of the skin and clothes of HCWs is needed because it is a key measure to interrupt nosocomial transmission of viruses like SARS-CoV to HCWs
- Several potential options exist that might prevent or reduce the risks of such contamination, including double gloving, use of surgical protocols for PPE removal, and PPE impregnated with an antimicrobial agent
- These potential alternative methods for PPE removal should be validated by testing using analytical methods such as those described for the human volunteer studies done using the CDC algorithm and protocol
- If deposited in high numbers, coronaviruses dried onto surfaces may survive for days at temperatures and humidity levels found in healthcare environments, with times for 99% reduction (1% virus survival) at 20°C and 50% RH approximately 2 days for TGEV and 3 days for MHV
- Overall, virus survival was enhanced by low temperatures; the relationship between temperature and inactivation rate was 4°C>20°C>40°C.
- In these experiments, the relationship between virus inactivation on surfaces and relative humidity level was not linearly correlated, which is consistent with observations made by other investigators of non-enveloped viruses dried on surfaces
- Results from this study suggest a roughly U-shaped relationship between virus survival and RH; compared to moderate (50%) RH, high (80%) RH has a protective effect for viruses dried onto surfaces, but lower RH (20%) may also have this protective effect
- Inactivation rates for TGEV and MHV at 20°C and 50% RH in this study are consistent with previous observations of SARS-CoV survival on a polystyrene surface, suggesting that TGEV and MHV may be suitable models for survival and inactivation of SARS-CoV on surfaces. In contrast, human coronavirus 229E may be inactivated much more quickly than SARS-CoV

- TGEV and MHV deposited in high numbers (10^4-10^5 MPN) survive on materials used to make PPE, with survival of 1% of initial viruses after 2 to 24 hours.
- Virus inactivation occurs more quickly on ceramic, laminate, plastic, and PPE materials than on stainless steel surfaces.
- Viral inactivation on surfaces may be due to a complex of mechanisms that include temperature-dependent inactivation, desiccation, effects of the air-water interface, and oxidation
- The results of this study suggest that TGEV and MHV could serve as conservative surrogates for modeling the risk of indirect personal contact and environmental transmission of SARS-CoV by healthcare surfaces and PPE items, and can be used in studies to determine ways to interrupt this route of exposure posing a risk of disease transmission

Future research

In order to choose a surrogate virus that accurately models the survival of SARS-CoV in the environment, the results of these studies using bacteriophage MS2 on PPE and the animal coronaviruses TGEV and MHV in water and on environmental surfaces need to be compared to those from SARS-CoV survival and transfer studies. More also needs to be done to understand the mechanisms of viral inactivation on surfaces at different temperature and humidity levels, and how these mechanisms contribute to differential survival of members of the *Coronaviridae* on surfaces. The use of GFP tagged viruses with confocal laser scanning microscopy might contribute to understanding of the partitioning behavior of viruses at the AWI and the effect of this partitioning on viral survival.

There are several alternative protocols for removing PPE that might be able to reduce the risk of viral contamination of the wearer. These methods could be compared to each other in a single study, using a randomized controlled crossover design. Subjects would be randomized to different removal methods groups, with the CDC method serving as the control group. To assess the effect of the level of contamination, different amounts of virus could be used for the initial inoculum. These PPE removal protocols should also be tested using an enveloped virus as a model for SARS-CoV, such as an enveloped bacteriophage. The methods developed for elution of viruses from PPE would first need to be tested with an enveloped virus to ensure that recovery efficiency is comparable to that of MS2 or otherwise considered of acceptable magnitude. The randomized controlled crossover design could then be used to assess transfer of enveloped viruses from contaminated PPE and compare it to that of non-enveloped viruses.

The evidence presented here on coronavirus survival in water is only part of the evidence base needed to assess the risk of future coronavirus transmission via contaminated water and fecal droplets and aerosols. TGEV and MHV show potential as conservative surrogates for the survival of SARS-CoV both in water and on surfaces. Studies of the survival kinetics and fate of these viruses in aerosolized droplets of contaminated water could be used to assess the risk of future SARS transmission via these routes.

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