Pandemic Pivot: Understanding Fate, Transport, Disinfection, and Host Dynamics of Environmental Viral Pathogens and Surrogates in the Era of SARS-CoV-2

Erika K. Keim

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Reading Committee:

John Scott Meschke, Chair

Lori Robins

David Beck

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Abstract

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Erika K. Keim

Chair of the Supervisory Committee:

John Scott Meschke

Department of Environmental and Occupational Health Sciences

Environmental contamination of pathogenic viruses is a common occurrence and leading to a number of skin, enteric, and respiratory infections in occupational, nosocomial, and community settings. Viruses with environmental transmission routes have varied abilities to survive and persist in environmental mediums to aiding in their transmission to susceptible hosts. With an on-going novel coronavirus pandemic, investigation on the environmental fate, survival, transmission, and containment of environmental viral pathogens has never been a timelier doctoral dissertation pursuit. The overarching goal of this dissertation was to **investigate survival, fate, infection, and viral-host dynamics of environmentally transmitted viral pathogens.** To do this I conducted four different lines of investigation using a range of viral pathogens and their surrogates.

Aim 1. Indirect environmental infection transmission routes including surface and fomite-based transmission are largely misunderstood, in need of additional research to better understand disease dynamics, and implement successful interventions, such as surface disinfection. Surface transmission of

viral pathogens- including SARS-CoV-2- is difficult to pinpoint in epidemiological investigations primarily because it is difficult to eliminate other transmission routes such as direct contact, droplet, and aerosol transmission. Therefore, the first aim of this dissertation was to investigate surface survival of coronaviruses and phage based on temperature, relative humidity, background matrix, and initial viral load. We found that Human Coronavirus OC43 (HCoV-OC43), Murine Hepatitis virus and *Pseudomonas* bacteriophage phi6 (phi6, enveloped) followed previously studied trends with increased die off with increasing temperature and relative humidity on stainless steel in culture. Additionally, we found that the addition of 1% mucin had a preserving effect for phi6, tripling the average time for a one $\log_{10}$ reduction to occur. This research bolsters and collates viral survival information on two coronaviruses and one enveloped phage and has implications for environmental health in the built environment.

Aim 2. Chemical disinfection is an effective way to reduce pathogen loads on surfaces. Previous research has shown that hypohalous acids HOCl and HOBr have been used as effective surface disinfectants on an array of different pathogens, but few studies have directly compared HOCl and HOBr in pure solutions. I conducted a direct comparison of HOCl and HOBr disinfectants at pH=5 on ~10^8 PFUs of MS2 in both suspension tests and dried on stainless steel surfaces. Suspension tests were conducted with 30 and 60 seconds of contact time and 60, 120, 240, and 480 µM HOCl and HOBr concentrations. Increasing concentrations and time led to increasing $\log_{10}$ reduction values of viable MS2. At 240 µM, HOCl and HOBr were able to achieve $\log_{10}$ reduction values of 3.45 ± 0.32 and 4.27 ± 0.67 (±95% CI) with HOBr significantly more effective at MS2 disinfection than HOCl. This was also true at 480 µM, where HOBr yielded a $\log_{10}$ reduction value of >7.42 ± 0.09 compared to HOCl at 6.31 ± 0.51. Disinfection comparison of MS2 dried on stainless steel coupons with 95, 190, 475, 950, and 1400 µM of HOBr or HOCl, found that HOBr was a more effective disinfectant at all concentrations. Additional work on species characterization and stability found that HOCl remained stable and pure for weeks at pH values less than five, while HOBr was less stable and degraded within hours of being made. These results have implications for use of hypohalous agents for surface disinfection to mitigate viral pathogens.

Aim 3. Animal research facilities that use recombinant viruses and viral vectors are held to a variety of regulations related to animal care, occupational health, and biosafety. Fecal waste derived from infected animals must be maintained in facilities that are cleaned daily creating ergonomic and biohazardous challenges for workers and the environment. To help address the issue fecal wastewater contaminated with recombinant viruses and viral vectors poses, chemical disinfection using potassium hydroxide and quaternary ammonium-based disinfectants were evaluated against a suite of seeded enveloped and non-enveloped viral pathogens and phages in animal research wastewater. Enveloped viruses phi6 and simian retrovirus-2 were readily inactivated (> 4-$\log_{10}$ reductions) through chemical disinfection. Non-enveloped viruses MS2 and Human Adenovirus-2 (HAdV-2) experienced minimal to no reduction through disinfection (<0.5 $\log_{10}$ reductions). To better understand partitioning of viruses within fecal wastewater, culture-based assessment of MS2 and phi6 revealed overwhelming partitioning into the liquid portion of samples. Using qPCR, HAdV-2 overwhelmingly partitioned into the liquid portion while HCoV-OC43 partitioned to solids. This research helps inform research facilities using recombinant viruses and viral vectors on the efficacy of chemical disinfection of wastewater. Partitioning results also have implications for SARS-CoV-2 wastewater surveillance.

Aim 4. Theiler's Murine Encephalomyelitis virus (TMEV) is an important virus used in biomedical research to induce multiple sclerosis and epilepsy in murine models. While this virus is readily used in biomedical research, there is a paucity of research on natural disease progression from the gut. Additionally, there is a viral-host interaction involving integrating host glutathione (GSH) into the capsids of public health relevant picornaviruses. To better understand host-viral interactions of TMEV, we first needed to determine how it infects the murine gastrointestinal tract and then determine its sensitivity to changes in host GSH. To do this, I developed a series of murine intestinal enteroid cell lines that had variable expression of host GSH while also representing several intestinal cell types. I also tested TMEV GSH sensitivity in monolayer BHK-21 cells by treating cells with a GSH inhibitor, buthionine sulphoximine, prior to infection. This research did not provide evidence for TMEV replication in murine intestinal enteroids and indicated that TMEV is not sensitive to environmental depletion of intracellular

GSH in monolayer. Future research should include more global approaches such as RNA-seq and *in vivo* experiments.

This research is far reaching and covers many topics including surface survival of pathogens, disinfection of viruses on surfaces and in wastewater, and viral-host interactions of a murine enteric virus. Work deriving from this research has far-reaching components within environmental public health, microbiology, and occupational health.

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Specific Aims

Environmental contamination of pathogenic viruses is a common occurrence and leads to a number of skin, enteric, and respiratory infections in occupational, nosocomial, and community settings. Viruses with environmental transmission routes have varied abilities to survive and persist in environmental mediums to aiding in their transmission to susceptible hosts. With an on-going novel coronavirus pandemic, investigation on the environmental fate, survival, transmission, and containment of viral pathogens has never been a timelier doctoral dissertation pursuit. This research analyzes a variety of pathogens in several case scenarios.

Indirect transmission of viral pathogens through surfaces represents a small but significant environmental transmission route in which pathogens can infect susceptible hosts via the mouth, eyes, or skin (Boone and Gerba 2007). While not always the primary routes of transmission, indirect surface transmission play a role in transmission of respiratory illnesses, including the novel coronavirus (National Center for and Respiratory Diseases 2020). While SARS-CoV-2 transmission has primarily been attributed to direct contact, droplet and aerosol transmission (Leung 2021), it is necessary to better understand all routes of transmission including surface survival and transmission. Other significant viral pathogens that are transmitted through indirect transmission via surfaces and fomites include enteric viruses (poliovirus, norovirus, rotavirus, and adenovirus) and respiratory (rhinovirus, RSV, measles, influenza) (Boone and Gerba 2007). Recorded infections occur in healthcare, community, and occupational settings and represent significant public health problems (Boone and Gerba 2007). Therefore, the overarching goal of this research was to **improve our understanding of the survival, fate, infection, and viral-host dynamics of environmentally transmitted viral pathogens to help with implementation of successful interventions and reducing transmission and burden of disease in susceptible populations.** To accomplish this goal, I addressed four specific aims within three broad topics.

Survival and Persistence of Enveloped Viruses on Surfaces

Frequent use of viral surrogates in applied environmental microbiology experiments have allowed researchers to understand fate, transport, persistence, and efficacy of interventions of target pathogens. These replacement microorganisms are freely used in method validation, internal controls, and disinfection studies because of their diminished or nonexistent pathogenicity, ease of growth, use as internal controls, and relative closeness to pathogens of public health importance. While these surrogate pathogens are useful, they are often not compared with each other leading to different results, confusing comparisons, and diminished capacity to make inferences on real pathogens of interest. This is especially proven true in studies using seeded internal controls for coronavirus recovery in wastewater and surface contamination (Li, Zhang et al. 2021). To address these concerns and lack of research on the comparison of surrogate viruses:

AIM 1. **I compared the survival of enveloped virus surrogates and human coronavirus strains on surfaces across temperature and relative humidity by looking at longitudinal changes in viral viability on stainless steel in tissue culture.** I hypothesized that *viral die-off will be greater at higher temperatures and RH on surfaces will increase longevity of infectious viral particles*.

This research substantiated previous knowledge of enveloped virus survival on surfaces and provided a valuable simultaneous experimental comparison between two coronaviruses and one enveloped surrogate virus used in environmental virology research.

Chemical Disinfection of Pathogens

Chemical disinfectants can decrease overall load of contaminating pathogens present in environmental media greatly reducing risk of transmission through surfaces, water, and wastewater. Mechanisms of viral pathogen inactivation varies greatly by disinfectant but involve disruption of critical membrane envelopes, proteins, enzymes, or nucleic acids necessary for environmental survival and transmission to susceptible hosts. Chemical disinfectants have been a ubiquitous part of cleaning and have a long history in reducing risk of viral infection across healthcare, occupational, and community settings.

Our research demonstrates that chemical disinfection can be effective but careful selection of disinfectants should be based on the pathogenic target and environmental conditions.

On surfaces, there is increased interest in HOCl and HOBr disinfectants to target pathogens over other chlorine-based disinfectant because they have demonstrated stability and can be easily tolerated on skin and mucus membranes. Previous research has also demonstrated effective disinfection using HOCl (Park, Boston et al. 2007), but no direct disinfection comparisons had been on HOCl and HOBr. To better understand capacity of each disinfectant on pathogen:

AIM 2. I evaluated the efficacy of hypohalous acids HOCl and HOBr as surface disinfectants on a pathogen surrogate on surfaces and in suspension based on time and concentration across two viral surrogates. *I hypothesized that HOBr will more efficacious than HOCl on viral surrogates on surfaces and in suspension compared to HOCl disinfection.*

Use of recombinant viruses and viral vectors in animal research facilities create a unique biohazardous waste scenario, where wastewater is frequently produced in animal research facilities. NIH guidelines require high levels of biosecurity and containment of any synthetic or recombinant nucleic acid research but provide little guidance on disposal (NIH Office of Science Policy 2019) prior to release into a sanitary sewer. Through a collaborative effort our research and the Washington National Primate Research Center looked at how chemical disinfectants can be employed to mitigate other non-biohazardous occupational hazards such as ergonomic and injury related injuries by employing washing, disinfection procedures of waste. This multi-layered approach to occupational biohazards can help to process biohazards when lifting and carrying wastes make autoclave sterilization hazardous for ergonomic reasons while protecting workers, the public, and the environment from recombinant viral pathogens. To disinfect infectious materials with large amounts of solids and demand:

AIM 3. I investigated a quaternary ammonium and pH raising potassium hydroxide disinfectant to mitigate enveloped and non-enveloped viral pathogens and viral surrogates in a primate animal research facility wastewater. I *hypothesized that there will be greater viral die-off of viruses at higher concentrations and contact times of both disinfectants but that non-enveloped viruses*

will be more resistant to disinfection and will not be significantly affected by high pH disinfection compared to no treatment.

Viral-Host-Environment Dynamics of Theiler's Murine Encephalomyelitis Virus

Development of infection after exposure to a viral pathogen in a susceptible host is dependent on numerous factors including cell type/function, immune responses, dose of virus received, route of exposure, microbiome, and many more. Understanding the viral-host mechanisms behind viral infection is important for understanding infection and disease progression but can also inform dose-response models and identify therapeutic targets. Additionally, environmental factors such as nutrient deficiency and toxic chemical exposure could alter viral-host dynamics by enhancing viral proliferation due to depressed immune responses or decreased viral replication due to insufficient cellular resources. These viral-host dynamics happen at the tissue and cellular level with exposure points at the lungs, skin, and GI tract for environmentally transmitted viral pathogens.

Intestinal enteroid cell culture models derived from intestinal stem cells can expand and proliferate to represent most epithelial cell types and physiology. These novel cell culture platforms provide a reductionist model of the intestinal epithelium to study host-pathogen interactions and are well known for their usage in the cultivation of human norovirus a previously uncultivable and fastidious human viral pathogen (Ettayebi, Crawford et al. 2016). One pathogen of interest was Theiler's Murine Encephalomyelitis virus (TMEV), a murine cardiovirus, because of its use in biomedical research to induce multiple sclerosis and epilepsy in murine models. While this virus is readily used in biomedical research there is a paucity of research on natural disease progression from the gut. Additionally, there is a viral-host interaction involving integrating host glutathione into the capsids of public health relevant picornaviruses (Ma, Liu et al. 2014, Thibaut, van der Linden et al. 2014). This interaction has been well established and cited for druggable targets (Duyvesteyn, Ren et al. 2020) and vaccine production (Abdelnabi, Delang et al. 2017), but has never been tested in the context of environmental depletion of

glutathione or using TMEV. To look at this potential interaction and test the ability of TMEV in a novel cell culture platform:

AIM 4. I examined the viral-host- environmental dynamics by characterizing an environmental interaction through the antioxidant system glutathione (GSH) that has been characterized in other picornaviruses and observing host interactions through viral exposure to murine intestinal enteroid cultures. *I hypothesize that TMEV replication would decrease with decreasing intracellular GSH by observing decreased infectious viral particle production in cells with depleted GSH, and that TMEV would robustly replicate in wild-type murine intestinal enteroids by observing increases in viral RNA over time.* My research demonstrated that TMEV is less sensitive to environmental depletion of intracellular GSH (Appendix A: TMEV Sensitivity to Host Glutathione) and has a more complicated viral-host-environment dynamic than previously known; as demonstrated by the unclear evidence of replication in wild-type enteroids (Evaluation of Theiler's Murine Encephalitis Virus in Intestinal Enteroid Cultures) furthering the need for additional research that incorporates immunological, microbiome, or host components.

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1. Literature Review Understanding the Use of Surrogate Viruses to Inform Environmental Surface Studies

Introduction

Infections resulting from fomite transmission of viral pathogens occur in almost all settings including agricultural, healthcare, occupational, educational, and community settings (Boone and Gerba 2007). Within these settings, infections from fomite transmission encompass a wide range of viral infections including respiratory, enteric, and skin in humans and animals. Evidence from detection of viral pathogens on environmental surfaces and suspected epidemiological case studies indicate fomitic transmission of viral pathogens is a relevant scientific field but fomite transmission is one of the least characterized transmission pathways and has been a source of confusion throughout the SARS-CoV-2 pandemic (World Health Organization 2020, Leung 2021).

The novel coronavirus, SARS-CoV-2 which causes the disease COVID-19, has caused the largest pandemic since the 1918 flu infecting over 177 million and more than 3.8 million deaths globally (Dong, Du et al. 2020) with wide ranging global effects including shrinking economies (Boettke and Powell 2021), exacerbation of other health issues such as psychological stress and suicide (Moriarty, Bourbeau et al. 2021), and drastically altered geopolitical landscapes (Chan, Gentile et al. 2020). Due to this there has been increased action among the scientific community to address its wide-ranging effects, where scientists have published and pivoted to research surrounding COVID-19 in almost all fields (Shapira 2020). While the primary transmission routes for SARS-CoV-2 are believed to be direct transmission related to close contact with an infected individual, aerosol or droplet transmission, there have been conflicting public health messaging and research surrounding fomite transmission and risk of viral infection (Schwartz 2021). Determining fomite transmission of viral pathogens such as SARS-CoV-2 is difficult to determine largely because other transmission routes are difficult to eliminate (National Center for and Respiratory Diseases 2020). There have been several direct studies on SARS-CoV-2 that that indicate fomite and surface transmission are possible due to the presence of viral RNA in public settings

(Harvey, Fuhrmeister et al. 2021) and experimental data demonstrating infection due to fomite transmission (Lee, Zhang et al. 2020, Port, Yinda et al. 2020); however, most surface related studies on SARS-CoV-2 have relied on the use of viral surrogates to make assumptions and public health recommendations concerning fomite transmission.

Surrogate viruses are viruses or bacteriophages are used in place of a single pathogenic virus or suite of pathogenic viruses of interest that have well documented environmental transmission routes (Boone and Gerba 2007). Surrogates for pathogens are called process organisms, indicators, tracers, or model viruses, are substitutes used in place of the actual pathogens for experimental purposes and in regulatory settings to help align and validate surface transmission, disinfection, or other scientific surface studies (Sinclair, Rose et al. 2012). Since SARS-CoV-2 has been designated for use at the BSL-3, reliance on surrogate viruses to conduct studies related to its transmission, survival, and various interventions has resulted in the use of more than eight different surrogates with little harmonization amongst the scientific community (Guillier, Martin-Latil et al. 2020, Pendyala, Patras et al. 2020). Surrogate organisms have been readily used in environmental microbiology as benchmarks for fecal contamination, water quality, and other environmental metrics for primarily bacteria and viruses.

Ubiquitous utilization of surrogate viruses for surface transmission of viral pathogens and SARS-CoV-2 has been useful across environmental virology research, but careful consideration of the surrogate is required. Through this review we aim to update and collate the usage of viral surrogates for surface and surface disinfection studies as replacements for viral pathogens, specifically in the context of the SARS-CoV-2 pandemic. There are two other reviews which extensively cover viral pathogens on surfaces (Boone and Gerba 2007) and the use of surrogate pathogens (Sinclair, Rose et al. 2012) but we will not go as in-depth and will have a more updated approach for surface and disinfection related research and criteria considerations with a heavy focus on SARS-CoV-2.

Survival, and Transport of Viral Pathogens on Surfaces

Pathogen deposition. Surface transmission of viral pathogens cannot occur unless there is a route of exit from the host into the environment. Contamination of surfaces is directly related to infection type, and manifestation of the viral illness through media such as aerosols, droplets, and bodily substances such as feces and vomitus.

Droplets and Aerosols. Respiratory viruses, such as SARS-CoV-2, rhinoviruses, and influenza A virus, are primarily deposited on to surfaces directly through droplets or aerosols via coughing and sneezing. Aerosol transmission can be differentiated from droplet spread in their ability to reach the respiratory tract of a susceptible individual which has largely been dictated by the size of droplets and particulate nuclei (Wells 1934). Droplets typically drop out of suspension rapidly, while aerosolized viruses can travel further (> 2m) either to susceptible hosts (Miller, Nazaroff et al. 2021) or depositing on surfaces. One study found definitive evidence of aerosolized SARS-CoV-2 viral RNA on surfaces in hospital ventilation systems where other mechanisms of deposition could be ruled out (Nissen, Krambrich et al. 2020). While this example is unlikely to cause fomite transmission, it highlights the ability of aerosols to deposit viral pathogens on surfaces in addition to other routes.

Droplet deposit is more predictable than aerosol deposition and is dictated by gravity, expulsion speeds, droplet size, and relative humidity of the environmental air during droplet generation. Droplet formation from feces has also been shown experimentally, with droplet generation of enteric virus surrogate (MS2 bacteriophage) and poliovirus-1 from toilet flushing was able to contaminate back walls, toilet paper dispensers, and floors of bathrooms (Gerba, Wallis et al. 1975, Sassi, Reynolds et al. 2018). Droplet deposition of viruses via vomit have been shown experimentally to distribute a norovirus surrogate (Feline calicivirus) as far away as 3 meters from the generating source making vomiting an especially efficient source for fomite transmission (Jones, Kramer et al. 2007, Kraay, Hayashi et al. 2018, Makison Booth and Frost 2019). Distribution of viruses on surfaces from droplets are found in higher concentration near generating sources (i.e. infected individuals, toilets) and high touch surfaces indicating indirect deposition from hands. Droplet to surface contamination has also been implicated in a number of

different norovirus outbreaks where vomiting was a primary risk factor for secondary cases (Chadwick and McCann 1994).

Direct contamination. Enteric viruses, such as noroviruses, hepatitis A, and rotaviruses, are directly or indirectly deposited on to surfaces via contaminated objects (i.e. hands, fingers, etc.) of vomitus or feces. Certain environmental conditions such as communal sanitation (Ho, Glass et al. 1989), and animal and livestock facilities (VanderWaal, Perez et al. 2018) can also have potential to spread fecal and respiratory derived viral pathogens through feet, hands, etc. Enteric viruses are especially efficient with fomite transmission due high shedding rates of infected individuals and low infectious dose of viral pathogens such as norovirus (Jones, Kramer et al. 2007). Systemic viral infections, such as Ebola virus, have been known to deposit viruses on surfaces from multiple sources and mechanisms. Saliva, stool, semen, breast milk, sweat, tears, and blood of Ebola infected patients were found with detectable virus with potential for deposition onto surfaces (Bausch, Towner et al. 2007). Due to the lack of aerosol distribution and short environmental survival of Ebola virus, viral presence on surfaces was more prevalent immediately adjacent to symptomatic patients and on protective equipment of caretakers (Poliquin, Vogt et al. 2016). Modelling of fomite transmission of various viruses have found that direct contamination is the most likely to lead to additional infections but that transfer efficiencies between fomites are sufficient enough to lead to an increased infection risk (Kraay, Hayashi et al. 2018).

Abundance in the environment. When surveilling surfaces and the environment for viral pathogens, studies find relatively low levels of pathogens. Typically, studies have focused on locations where viral pathogens are expected to have higher circulation by sampling hospitals (Cheesbrough, Green et al. 2000), schools (Fong, Leung et al. 2020), offices (Stobnicka, Gołofit-Szymczak et al. 2018), homes with infected individuals (Amin, Stowell et al. 2018), and/or sampling during seasonal influxes (Boone and Gerba 2005) or pandemic spread (Harvey, Fuhrmeister et al.). Methods used for pathogen detection on surfaces are almost exclusively molecular detection methods such as PCR, qPCR, RT-PCR, etc., where detection of viral nucleic acid is highly specific and able to detect low levels of viral contamination. These methods are often specific to the tens of genome copies per reaction on surfaces

(Ganime, Carvalho-Costa et al. 2012) providing valuable insight on environmental transport and dynamics in these settings. While these methods are readily and easily deployed at a large scale, they provide no information on pathogen viability and understanding the risk of infection interpreted from molecular methods is an on-going field of study (Rodríguez Roberto, Pepper Ian et al. 2009). Stated abundances of viruses on surfaces range significantly, but typically follow important patterns based on the prevalence in the population (Harvey, Fuhrmeister et al. 2021) and the presence of an infected individual (Amin, Stowell et al. 2018).

Viral Pathogen Survival. Viral survival on fomites and surfaces can be broken into two broad categories of intrinsic and extrinsic characteristics of the pathogen.

Intrinsic characteristics of viral pathogens are based on viral structural components of capsid proteins, genome characteristics, enzymes, repair mechanisms, and sometimes a lipid membrane envelope. Viruses without membrane envelopes are more rigid and resistant to environmental elements with many enteric viruses as examples of hearty viruses without membrane envelopes (Howie, Alfa et al. 2008, Firquet, Beaujard et al. 2015). Viral genomes come in many shapes and sizes. Viruses on the Baltimore classification system are divided up into viruses with DNA and RNA genomes and further based on single or double strandedness, genome sense, and segmentation. While strict intrinsic integrity based on genome this can be especially important when considering environmental sources of nucleic acid degradation, UV light or radiation-based interventions (Lytle and Sagripanti 2005). Pyrimidine base pairs resist UV-C degradation an order larger than purine bases (Rockey, Henderson et al. 2021). Repair functions, in the form of host enzymes and repair polymerases, allow for some physical damage of the virus but will maintain viral viability and repair itself after entry into a host cell. Adenovirus is a non-enveloped DNA virus that is well known to resist UV disinfection because of its double stranded DNA genome and ability to use host repair enzymes that aid its repair after entry into host cells (Day 1974, Rainbow 1974).

Extrinsic factors related to fomite survival range from host factors, surface types, background matrices, and environmental conditions. Surface type and material is an extrinsic factor of viral survival

that is well studied in environmental microbiology aiding in decision making related to surfaces found in foodservice, healthcare, and other points for pathogen transmission. Experimentally, surfaces that are porous have lower recovery efficiencies and have lower transfer rates non-porous surfaces (Liu, Brookbank et al. 2020). Porous surfaces also have wicking and moisture absorbing properties that increase pathogen adherence to fomite. Experimentally avian influenza (Tiwari, Patnayak et al. 2006), phi6 phage (Whitworth, Mu et al. 2020) and enteric viruses (Abad, Pintó et al. 1994) all had increased die-off on porous materials such as curtains, cloth, and paper compared with non-porous materials such as aluminum, stainless steel, latex, glazed ceramics, and eggshells. The exception in these studies was avian influenza viral die-off on feathers was comparable to other non-porous surfaces tested (Tiwari, Patnayak et al. 2006). All experimental studies have cited recovery efficiency and desiccation differences in surface materials as influencing factors in survival of viral pathogens on surfaces. From a control perspective, non-porous materials are more easily disinfected and sterilized for reuse compared with porous materials without concern for residual viral pathogens but added care must be taken because of increased longevity of viral pathogens on these surfaces.

Background matrices and environmental conditions influences both desiccation conditions and die-off of viral pathogens after deposition onto surfaces. Environmental conditions such as pH of suspending media (Lin, Schulte et al. 2020), temperature (Morris, Yinda et al. 2020), relative/absolute humidity (Sattar, Karim et al. 1987, Casanova Lisa, Jeon et al. 2010), and presence of bodily fluids (Schuit, Miller et al. 2016) have all been shown to influence viral die-off in the environment. Humidity has a strong but reverse influence on non-enveloped enteric and enveloped viruses. Non-enveloped enteric viruses had increased die off in low humidity environments and increased longevity in moist environments (Sattar, Karim et al. 1987). The noted exception to this was hepatitis A which had greater longevity at lower relative humidity (~95%) compared with polivirus-1 (Mbithi, Springthorpe et al. 1991). Enveloped viruses survive better in lower humidity environments but increasingly die-off in high humidity (Casanova Lisa, Jeon et al. 2010). Seasonality of influenza, an enveloped virus, in Northern and Southern hemispheres, as well as a lack of seasonality in equatorial regions, is partly due to

environmental survivability of flu viruses based on regional relative humidity and temperature changes (Marr, Tang et al. 2019). This same seasonality in influenza is also expected to become the expected pattern of seasonal SARS-CoV-2 (Smit, Fitchett et al. 2020, Rojas, Cordo et al. 2021). In contrast, non-enveloped enteric viruses survive much longer suspended in liquids and with increased air humidity due to high water content of fecal material, routes of exit, and large pH variations (Taylor, Moore et al. 1981) in the gastrointestinal tract. Poliovirus readily aggregates at neutral pH increasing long term survivability, but will disaggregate above pH ~9 still maintaining some viability but with increasing die-off (Gassilloud and Gantzer 2005). Both high and low pH conditions were found to be virucidal to avian influenza where die-off at neutral pH was greater than 24 hours but was reduced to less than 6 hours at extreme pH conditions (Shahid, Abubakar et al. 2009). Similar results were seen with the enveloped phi6 bacteriophage where extreme pH variations in suspending material had greater impact on viability compared to the non-enveloped MS2 bacteriophage (Lin, Schulte et al. 2020).

Background matrices such as fecal material, vomit, blood, saliva or bodily fluids can increase viral aggregation, and have a preserving effect on viral pathogens (Jean, Morales-Rayas et al. 2011, Schuit, Miller et al. 2016). Experimentally, surface experiments can look at matrix effect where viral cultures of high titer and purity can be mixed with standardized background matrices using porcine or bovine mucin, tryptone, yeast extracts, tissue culture media, or sera to help mimic various bodily fluids (Sattar, Springthorpe et al. 2003). In two separate SARS-CoV-2 fomite studies, added mucin, bovine serum albumin, and tryptone increased viral longevity on Tyvek PPE clothing from hours (half-life= 6.74-10.05 hours) (Haddow, Watt et al. 2020) to three weeks (21 days) (Kasloff, Leung et al. 2021) demonstrating how protective bodily fluids can have on viral survival on fomites. Background matrices have the effect of retaining moisture and prolong the drying process on surfaces providing some protection from desiccating forces which can increase die-off even in viruses. The caveat to this would be in environmental conditions with significant fungal and microbial backgrounds, microbial predation is cited as a significant factor for increasing die-off for viruses in wastewaters, latrines (Karim, Glenn et al.

2008), and foods (Nasheri, Harlow et al. 2021) and could play a part in surfaces that are highly contaminated with biologically active background matrices.

Surface Disinfection of Viral Pathogens

Environmental durability of microorganisms has been largely defined by the Spaulding Classification which was developed in 1957 and has been continued to be used make disinfection and control choices for environmental viral pathogens for semi-critical and critical surfaces and equipment (Spaulding and Emmons 1958). This classification system relies on the assumption that the level of disinfection working at the tested level and all microbes below it on the hierarchy of microbial durability. While this system is still incredibly relevant for environmental microbiology, our work and others have found that viral pathogens can be resistant to disinfection mechanisms that are effective against other pathogens that are higher up on the hierarchy (Solomon, Fino et al. 2009). It's important to highlight both differences in biology between viruses and bacteria and the differences in disinfection mechanism when choosing infection control methods. Others have proposed different hierarchies of microbial resistance based on viral and bacterial pathogens (Sattar 2007). This more hierarchical approach to viral disinfection proposed by Sattar reflects the nuanced approach to viral disinfection compared with other broad classes of pathogens (i.e., bacteria, fungi) and prioritized non-enveloped viruses for separate disinfection susceptibility testing alongside bacterial spores and fungal pathogens.

From a regulatory standpoint, US EPA and FDA regulate all antimicrobials used in the environment, handwashes, hand rubs, and topical antiseptics in the United States. US EPA federal regulations require that disinfectants meet testing standards prior to labeling and that all antimicrobial claims be made under certain testing guidelines. Results from validation studies inform labeling instructions and can certify disinfectants for two major types: 1) hospital types which include medical and dental instruments, floors, toilet seats and other surfaces, and 2) general use disinfectants for households, swimming pools, and water purifiers. Disinfectants with virucidal claims through the US EPA must demonstrate a 5-log10 for bacteria and completely inactivate target virus or viral surrogates with a

minimum of 3-$\log_{10}$ reduction if cellular cytotoxicity cannot be overcome in testing (US EPA, OCSPP 810 series) (EPA 2018). Labeling claims can require disinfectants to be effective under various conditions including viral/pathogen levels, within a certain amount of contact time, concentration, and inclusion of testing with organic matter, soil, or sera (Sattar, Springthorpe et al. 2003, Sattar 2007). FDA regulations regarding hand soaps, antiseptics, and hand rubs for healthcare and over the counter use require no viral disinfection testing, but as recently as 2019 implemented an increased $\log_{10}$ reduction threshold for bacteria for most regulated products (Food and Drug Administration 2016). Regulation and registration of chemical sanitizers and disinfectants are required to demonstrate antimicrobial activity they are not required to demonstrate a reduction in infection. This is an important distinction because their application in settings will not always be effect across different transmission types and can be difficult to demonstrate if other disinfectants are in use. In the European Union, disinfectants and antimicrobial products are regulated by the Registration, Evaluation, Authorization, and Restriction of Chemicals legislation (commonly referred to as REACH) through the European Chemicals Agency.

Direct testing of disinfectants for antimicrobial is important to demonstrate direct effects on viral pathogens. These results are required for regulatory purposes but also provide important research information concerning the susceptibility of viruses to disinfectants. For surface testing, products are evaluated either in suspension (ASTM E2315: Liquid suspension Time-Kill Test) or dried on surfaces (ASTM E2197: Standard Quantitative Disk Carrier Test Method, EPA Test Guidelines 810 Series, or as described by Sattar, et al 2003). Standardized methods can help determine disinfectant concentration and contact time needed to achieve desired viral reduction. Viruses that are tested using these disinfectants can be target pathogens for the disinfectant. A certain number of surrogate viruses are frequently used for labeling purposes as well with feline calicivirus replacing human norovirus, bovine viral diarrhea virus for hepatitis C, or duck hepatitis B in place of human hepatitis B (EPA OCSPP 810 series). This can be helpful for regulatory and labeling purposes if the seller wants to market their product as being efficacious for certain pathogens but are unable to use the actual pathogens. While these are standardized requirements for EPA guidelines, there is evidence that feline calicivirus as a surrogate for human

norovirus not comparable for disinfection as it is more labile than other caliciviruses, where other caliciviruses such as the Tulane virus would be a more conservative surrogate (Cromeans, Park Geun et al. 2014, Kniel 2014). This choice in surrogate has largely been driven by the lack of tissue culture system for human noroviruses, even with improved and updated disinfection research on caliciviruses this EPA surrogate remains inflexible. Other considerations for disinfectant testing are chemical compatibility with surface type (i.e., bleaching agents, pitting), consumption of disinfectant due to background matrix (i.e., chlorine, oxidants), toxicity to users, and feasibility of application.

In practice and research, application of chemical disinfectants can interrupt transmission and reduce viral infections by reducing viral loads on surfaces (Tuladhar, Hazeleger et al. 2012). Many interventions are well integrated into high transmission settings such as foodservice, hospitals, and school/daycare settings. Epidemiological evidence demonstrates that added hygiene and disinfection measure reduce infections, but overall contributions fomite transmission and surface disinfection can be difficult parse out due to the role of other transmission routes, low disease prevalence, multi-level interventions, or ubiquitous uptake in hygiene/disinfection practices. Two Quantitative Microbial Risk Assessment studies of viral fomite transmission have found that hand-to-face activity contributes significantly to fomite transmission but that a single intervention, such as hand washing, is unlikely to stop all fomite based infections because of low infectious doses and efficient transfer rates of viral pathogens (Kraay, Hayashi et al. 2018).

In experimental trials, introducing hand sanitizers into office settings resulted in fewer reported respiratory illnesses in employees. Hygiene interventions such as providing hand sanitizers and facial tissues were shown to reduce transmission of a viral tracer on hands and fomites in an office setting (Sassi, Sifuentes et al. 2015, Reynolds, Beamer et al. 2016). Several meta-analysis studies found that more frequent hand hygiene can reduce and identify efficacious interventions across all pathogens (bacterial, viral, etc.) (Wong, Cowling et al. 2014). One 2008 meta-analysis found that improved hand hygiene resulted in a 31% reduction (95% CI: 19-42%) in reported GI illness and a 21% reduction (95% CI: 5-34%) in reported respiratory illnesses, where the most benefits were seen in non-antibacterial hand

soap and water (Aiello, Coulborn et al. 2008). Hand hygiene interventions that looked at confirmed community-based influenza cases found that hand hygiene with or without face masks could reduce cases by 18% (95% CI: 0-34%) (Wong, Cowling et al. 2014). A systemic meta-analysis that looked at hospital surface disinfection protocols and rates of nosocomial infection, found that there was no decrease in nosocomial infection rates when disinfectants were used in addition to detergent-based cleaning products (Dettenkofer, Wenzler et al. 2004). Epidemiologically, reduction of infections rates due to surface disinfection and hygiene interventions are difficult to parse out likely due to the role of multiple transmission routes but still indicate promising reductions in infections through hand hygiene and surface disinfection measures.

Surrogates in Research

Surrogates are alternatives to target viral pathogens used in research when barriers to using the actual pathogen are present. While surrogate viruses are used in place of a target pathogen or used to generalize about a suite of viruses (i.e. enveloped RNA viruses, enteric, etc) they usually come in the form of actual viruses, phage, chemical or physical compounds that are used in research because they mimic or can provide information on the behavior of actual viral pathogens. Surrogates are also called several names based on the type of experiment and research being conducted and will be referred to in research as tracer, indicator, process, model, substitute or validation virus (Sinclair, Rose et al. 2012). These surrogates are replacements for actual viral pathogens with the intent to make characterizations for the behavior, survival, destruction, or transport of actual viral pathogens in research. They have been incredibly valuable to research and are well integrated into regulatory processes (FDA, EPA) for validation of disinfectants, sanitizers, and other microbial methods for environmental virology, but there needs to be stringent rationale and validation prior to using a surrogate instead of target pathogens.

Bacteriophage. Surrogates that are most used for environmental virology studies are primarily bacteriophages and mammalian viral pathogens, but other non-viral or microbial surrogates have also been used in specific studies or for educational purposes. Bacteriophages are commonly used in

environmental studies to replace Caliciviruses, Enteroviruses, Rotaviruses, Adenoviruses, and Astroviruses that are known to cause acute gastroenteritis, respiratory symptoms, diarrhea, and vomiting. Common bacteriophages surrogates include MS2, phiX174, T4, PM2 (Turgeon, Toulouse et al. 2014), PP7 (Ganime, Leite et al. 2015), PR772 (Gall, Shisler et al. 2016), and phi6 (Whitworth, Mu et al. 2020) . These have all been used in either surface survival, transport, or disinfection studies as substitutes to pathogenic viruses.

Non-Human Mammalian Surrogates. Animal viruses have also been readily used as replacements for human pathogens. A variety of animal coronaviruses including but not limited to murine hepatitis virus, bovine coronavirus, Transmissible gastroenteritis virus (TGEV), Canine coronavirus, and Porcine epidemic diarrhea virus have been used as surrogates and comparators to SARS-CoV-2, SARS-CoV-1, and MERS for surface related intervention and survival research (Guillier, Martin-Latil et al. 2020). Human norovirus is often substituted with animal Caliciviruses such as feline calicivirus and murine norovirus in research and regulatory validation studies (Wang, Hirneisen et al. 2013, Arthur and Gibson 2015, Makison Booth and Frost 2019). Mengo encephalitis virus (Drouaz, Schaeffer et al. 2015) and Theiler's Murine encephalitis virus have also been used or proposed as a poliovirus and enterovirus surrogate especially as poliovirus is eventually phased out of environmental microbiology research due to global eradication and its addition to select agent lists (World Health Organization 2015).

Human Viruses as Surrogates. In addition to animal viruses, human pathogens that are closely related to more consequential human pathogens are also used as surrogates to make inferences for behavior or interventions of viral surfaces. While these are almost always strict pathogens that cause disease in humans and present pathogenic risks to researchers, they tend to be limited to laboratory-based settings and almost never used in public facing environmental studies (i.e., transport studies). Human coronaviruses NL63, 229E, and OC43 have all been used and included in studies that either compare across respiratory coronaviruses and/or make inferences to pandemic SARS-CoV-2 and also the diseases they cause (Warnes Sarah, Little Zoë et al.). Human poliovirus has become the de facto prototypical enterovirus while not an explicit surrogate, results from these studies are broadly applied to all

enteroviruses, including Hepatitis A, with the expectation that all enteroviruses would react and behave the same in the environment and on surfaces (Richards 2012).

Types of studies that use surrogates often include validation studies looking at methods or processes, survival/die-off studies related to environmental conditions or various interventions such as cleaning processes, disinfection, etc, transport studies, internal controls to help validate environmental sampling and processing, and others.

Rationale for Choosing Surrogates over Viral Pathogens

Usage of surrogates in place or alongside viral pathogens of interest in research makes it clear there are a variety of limitations and rationalizations for using surrogates over target viral pathogens and making more accurate observations about the survival, behavior, or control of viral pathogens. Typically, surrogates are used for multiple reasons with some level of validation in comparison to the target virus. Validation criteria for surrogates has been outlined and proposed in across a number of different literature reviews but are uniquely related to the purpose and type of research being conducted (Sinclair, Rose et al. 2012).

Biosafety. Administrative restrictions on pathogens related to biosafety takes pathogens rated in risk groups 2 and 3 and replaces them with a non-pathogenic risk group 1 or less pathogenic risk group 2 pathogen. This can allow for environmental process or transport studies outside of a lab or biosafety cabinet where higher risk group pathogens requiring higher levels of containment (i.e. BSL-2, BSL-3) do no risk infecting research personnel or the public increases. The WHO, CDC, and other administrative bodies have specific facility requirements for research on select agents and risk group 3 pathogens regarding containment, disposal of biohazards, ventilation, restricted access, training, and verification prior to being able to use these pathogens in environmental surface research. In addition to administrative restrictions on certain pathogens, researchers might find added biosafety procedures to be a barrier in initiating research. Surrogates to viral pathogens allow for pilot data to be generated or completely replace the target pathogen if it has been well validated.

Virulence and pathogenicity. Along with administrative classification of biosafety, ability to cause disease of a target viral pathogen will determine if a surrogate will guide research decisions. High consequence viral pathogens with no known vaccine or treatment (i.e., Ebola, hemorrhagic fevers) will likely be replaced by a surrogate and reliance on previously studied surrogates. Similarly, less pathogenic and more readily accessible human coronaviruses (NL63, 229E, OC43) have been used to replace pandemic SARS-CoV-2 in numerous survival, disinfection, transport, and validation studies to make recommendations and inferences on the pandemic (Warnes Sarah, Little Zoë et al.). These have been useful trade-ins when access and availability of select agents and highly pathogenic viral pathogens are difficult to obtain or pose a great risk to researchers.

Ease of growth and concentration factors. Bacteriophages are readily used as surrogates because they can be easily grown (usually less than 18hrs) and have large burst sizes with stocks reaching high as 10^{14} per milliliter without additional concentration. Since bacteriophage hosts are relatively easy to grow compared to mammalian cell lines, phage have been favored as viral surrogates for studies that include large scale environmental studies to smaller pilot studies for an array of environmentally transmitted viruses (Reynolds, Beamer et al. 2016). On the other hand, human norovirus represents a large portion of viral gastroenteritis virus globally but was not cultivated *in vitro* until 2015 (Ettayebi, Crawford et al. 2016). Since 2015, there are three validated culture methods for human norovirus: human B cells (Jones, Grau et al. 2015), human intestinal enteroids (Ettayebi, Crawford et al. 2016), and zebrafish larvae (Van Dycke, Ny et al. 2019). While the development of these methods represent progress towards understanding one of the most prevalent enteric viruses, additional work needs to be done to develop feasible norovirus methods for environmental microbiology studies. Ability for researchers to cultivate and quantify viable virus provides a level of certainty in research observations concerning viral surface contamination and transport.

Ability to differentiate in the environment. Surrogates with unique growth signatures or distinguishing methods are used in place of target viral pathogens. Poliovirus receptor specific cell line L20B has been used in poliovirus environmental wastewater studies to help differentiate it from other

enteroviruses present in highly contaminated samples (Lodder, Buisman et al. 2012). Likewise, radiolabeled phage have been used as a recovery control for concentration methods for surveying oceanic viruses to gauge recovery and validate samples for further analysis (Noble and Fuhrman 2000).

Genetic similarity. Surrogate viruses within the same taxonomic genera, family or even order are regularly substituted for closely related viral pathogens. Animal coronaviruses such as murine hepatitis virus, bovine coronavirus, porcine coronaviruses, and canine coronaviruses are all examples that have been used for environmental microbiology research with implications for pandemic coronavirus (Guillier, Martin-Latil et al. 2020). While genetic similarity is often used to select surrogates, the premise is based on the phenotype of the surrogate being similar enough to the target viral pathogen that research results would otherwise be undistinguishable.

Environmental performance. Viral surrogates chosen based on overall structure, integrity, or disinfection resistance are also reasons researchers choose different surrogates and indicators. It has been noted that choosing surrogates for validation must behave and survive as long if not longer than the target organisms. Many researchers choose to use surrogates that are slightly more resistant to environmental degradation, disinfection, or destruction compared to their target pathogen in order to make conservative recommendations for interventions designed to reduce infection transmission risk (Wang, Hirneisen et al. 2013). In the case of human norovirus, surrogates can only be compared to each other where feline calicivirus and murine norovirus have noted differences in disinfection resistance and environmental survival (Cannon, Papafragkou et al. 2006, Park and Sobsey 2011).

Conclusions & Limitations of Viral Surrogates

Throughout the SARS-CoV-2 pandemic and other viral outbreaks, fomite transmission is difficult to track epidemiologically, but a likely occurrence and an important line of research to better understand public health implications. Use of surrogates in place of viral pathogens has greatly advanced environmental microbiology research but is an on-going line of inquiry to better validate and use them appropriately. With growing uncertainty of virus transmission in the beginning of the pandemic,

numerous research studies and literature reviews surrounding surface-based interventions, transmission routes, and environmental survival have dominated research spaces. At the time of this submission, PubMed articles concerning “virus, environment, fomite” produced 59 and 50 articles, reviews and commentaries for 2020 and 2021, respectively; whereas, 2019 had only 18 articles of the same nature . While more information has come out suggesting that fomite transmission represents a small portion of new infections, there is important evidence that fomite transmission is possible (Harvey, Fuhrmeister et al. 2021) resulting in less severe infections (Lee, Zhang et al. 2020, Port, Yinda et al. 2020). As the SARS-CoV-2 pandemic gets more under control via vaccination and increased healthcare capacity, continued circulation of subclinical infections could be driven by fomite transmission in food service, hospital, and community settings. Surrogate research on SARS-CoV-2 and all viral pathogens have greatly advanced environmental microbiology, but careful validation of surrogates is needed for effective hygiene and disinfection interventions, and to understand environmental behavior.

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2. Enveloped Virus Surface Persistence Comparison

Introduction

Pandemic SARS-CoV-2 has drastically changed the way scientific research has been conducted with a record number of researchers pivoting to address pandemic related needs within their various fields (Shapira 2020). Surface transmission of viral pathogens is well documented for a number of human and animal viral pathogens including norovirus (de Wit, Widdowson et al. 2007, Jones, Kramer et al. 2007) and avian influenza (Conraths, Sauter-Louis et al. 2016), but less is understood about the role of surface transmission of SARS-CoV-2 as a minor route of transmission. Airborne, droplet, and direct contact routes are largely attributed to SARS-CoV-2 transmission (Leung 2021) but minor transmission routes are difficult to attribute and there are only suspected in a handful of outbreaks (National Center for and Respiratory Diseases 2020, Schwartz 2021). Surveillance of high touch surfaces in community settings found that the presence of SARS-CoV-2 RNA was rare (8.3% of all samples) but longitudinally corresponded with local caseloads (Harvey, Fuhrmeister et al. 2021). Hospital and healthcare settings prevalence of viral RNA was present at low levels on surfaces around infectious patients such as bedside equipment, caretaker PPE, floors, and air vents indicating dispersion into the environment (Ben-Shmuel, Brosh-Nissimov et al. 2020, Wei, Huang et al. 2020, Zhou, Yao et al. 2021). Use of molecular methods for environmental surveillance is highly accurate in that they can provide evidence of pathogen deposition and dynamics on surfaces, but lack of culture-based methods mean that risk of transmission is uncertain and must be further explored to understand this in public health contexts.

Experimentally, two separate studies found fomite transmission of SARS-CoV-2 in hamsters led to less severe or subclinical infections (Lee, Zhang et al. 2020, Port, Yinda et al. 2020) indicating when SARS-CoV-2 is dispersed into the environment coming into contact with susceptible hosts via fomites infections can occur. To better understand indirect route of transmission via surfaces of SARS-CoV-2 and develop effective interventions further research is merited to address the survival and inactivation on surfaces.

Fomite transmission relies on the survival of SARS-CoV-2 on surfaces with viral inactivation being dependent on a variety of environmental conditions including relative humidity, temperature, (Casanova Lisa, Jeon et al. 2010) and background matrix (Rockey, Arts Peter et al.). Meta-analysis modelling research has shown a non-linear relationship between viral survival on surfaces and temperature across alpha- and beta- coronaviruses, where viral survival improves at low or 100% relative humidity, making the virus more resistant to thermal inactivation at extreme relative humidity (Guillier, Martin-Latil et al. 2020). Experimental research has found that SARS-CoV-2 is more stable on non-porous surfaces where at 22 °C and 65% humidity SARS-CoV-2 remained viable on stainless steel experiencing one $\log_{10}$ reduction in viability after 24 hours (Chin, Chu et al. 2020). A similar study found that it only took 11 hours for that at 27 °C and 65% relative humidity (Morris, Yinda et al. 2020) for SARS-CoV-2 to achieve the same $\log_{10}$ reduction value. These important studies highlight variability in study results and conditions but demonstrate the need for additional data on coronavirus survival on surfaces.

SARS-CoV-2 is currently categorized as biosafety risk group 3, where BSL-3 precautions limit its use across research making it difficult to better understand surface transmission in experimental settings. Surrogates can serve as alternatives to pathogenic microorganisms to understand fate, transport, disinfection, and survival of related or similarly structured or related pathogens in experimental settings. Coronavirus surrogates have rapidly replaced the need for BSL-3 settings applied microbiology research with as many as eight or more viruses being used in research (Guillier, Martin-Latil et al. 2020). In order to make scientific inferences about the transmission of pathogens and design effective interventions to mitigate their transmission, validation of surrogate viruses is necessary (Sinclair, Rose et al. 2012). Therefore, as part of this research we have selected three of the most prominent surrogates in the literature for SARS-CoV-2: Human Coronavirus-OC43 (HCoV-OC43), Murine Hepatitis virus (MHV), and *Pseudomonas* bacteriophage phi6 (phi6) to test their ability to survive in relation to temperature, relative humidity, and background matrix on stainless steel surfaces.

Methods & Materials

Viruses and host organisms. Bacteriophage phi6 and *Pseudomonas syringae* pathovar phaseolicola host were generous gifts from Dr. Krista Rule Wigginton from the University of Michigan, Department of Civil and Environmental Engineering. Phi6 was prepared by inoculating an overnight broth culture of log-phase *P. syringae* at an MOI= 0.02 and allowing for it to incubate on a shaking incubator 25°C overnight until broth clarified and bacterial debris precipitated out of suspension (~18 hours). Stocks were then centrifuged at 2,500 g for 5 minutes to pellet bacteria and supernatant was then filtered through a 0.22 µm filter and stored at -80° C until use. Phi6 phage stocks and samples were quantified by plating 100 µl of relevant dilutions of a sample in PBS using the double agar layer method (Adams 1959). Plates were incubated overnight at 25°C and enumerated as PFU/ml.

HCoV-OC43 stocks were prepared and quantified according to previously described methods (Philo, Keim et al. 2021). All culture-based samples were quantified by TCID50 method using the Spearman and Karber algorithm (Hierholzer and Killington 1996).

Murine Hepatitis virus A59 and murine delayed brain tumor (DBT) cell line were both generous gifts from Dr. Krista Rule Wigginton from the University of Michigan, Department of Civil and Environmental Engineering. DBT cells were grown in 10% FBS (ATCC) and DMEM supplemented with L-glutamine (Gibco) and sub-cultured every 3-4 days using 0.25% trypsin-EDTA (Gibco) as a cell dissociation agent. MHV stocks were produced by infecting a confluent monolayer of DBT cells in 10% FBS and DMEM supplemented with 1% Gen/Kan and incubated at 37 °C with 5% CO_2 until CPE was visible after 24 hours. Infected flasks were then frozen, thawed, and centrifuged at 3000g for 10 minutes at 4 °C. Supernatant was collected and filtered through a 0.22 µM filter, stocks (~10^6) were stored at -80 °C until use in persistence experiments.

For plaque assay enumeration of MHV stocks and samples, samples were diluted 10-fold in DMEM and 200ul was applied in duplicate to confluent DBT cells in 6-well tissue culture plates, cells and samples were incubated for 1 hour with rocking every 10 minutes prior to overlaying with 1.5%

Avicel 1:1 mixed with 2x MEM (Sigma) containing 1% Gen/Kan, L-glutamine, Nystatin, 10mM HEPES, sodium bicarbonate, and NEAA. Infected plates were incubated at 37 °C with 5% CO_2 for 48 hours prior to fixing with crystal violet and 20% Methanol and washing using PBS. Each plaque assay was performed with duplicate wells with at least one serial 10-fold dilution of viral stock was used as a positive control and one negative media control.

Surface persistence trials. Phi6 stocks were diluted 10-fold and filtered through a 0.22 µM filter and then either diluted in PBS or PBS with bovine mucin to provide a 1% mucin background. Due to low stock concentrations of OC43 and MHV there were no alterations to suspending media. 20ul of prepared virus inoculum was added to stainless steel discs coupons (Cat # RD128-316, BioSurface Technologies Co, Bozeman MT) and allowed to dry for 1hr in a type II BSC.

After drying, one negative control and three inoculated coupons were immediately eluted in 1mL of elution buffer which was 3% beef extract (phi6) or cell media (RMPI-1640 or DMEM) with 2% FBS and 1% gentamycin/kanamycin to determine initial viral load. The rest of inoculated triplicate coupons were placed into plastic container "microenvironments" with 8-12g of a saturated salt solution and temperature and relative humidity logger (Elitech RC51H) and placed into incubators set at 25 °C and 37 °C. Relative humidity of each microenvironment was controlled within 8% using saturated salts to maintain 23% (potassium acetate), 43% (potassium carbonate), or 65% (sodium chloride) at 25 °C and 37 °C. At relevant time points, inoculated coupons were processed by vortexing coupon with 1ml of elution buffer for 3 minutes and retaining liquid for culture analysis. For coupons with phi6, 3% beef extract was used to elute coupons. Elution buffers for OC43 and MHV were RPMI-1640 or DMEM, respectively, with 2% FBS and 1% gen/kan. Eluted samples were collected and stored at -80 °C until analysis by culture.

Reduction values from culture results were calculated using the following equation to get log10 reduction values:

$$Log_{10}\, Reduction\, Value = \; Log_{10}\left(\frac{C_0}{C_i}\right)$$

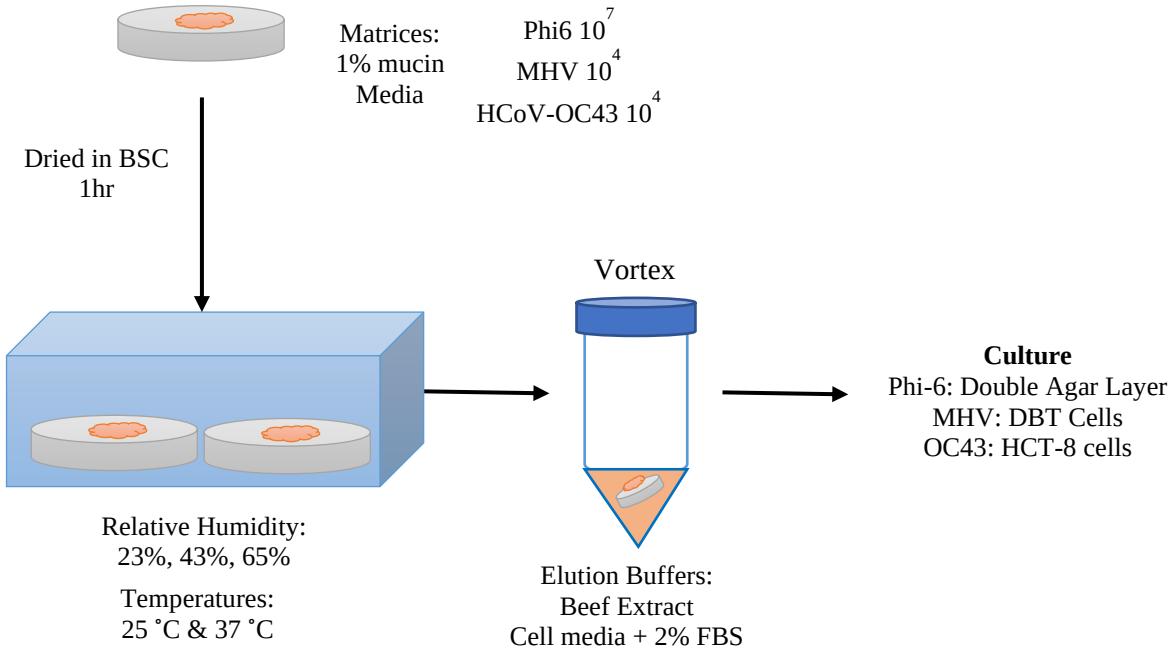

Figure 2.1 Methodological schematic of viral persistence experiments using stainless steel discs with Phi-6, MHV, or OC43 dried in a BSC.

Where the Log_{10} Reduction Value is calculated based on the initial load after drying (C_0) against the viral concentration (C_i) after i hours. Log_{10} reduction values were then visualized and subjected to simple linear regression to determine estimate T90 values using in GraphPad Prism version 9.0.2 (161) (San Diego, CA). Estimated T90 values were compared with graphical results of each virus and experiment, estimated T90 values from simple linear regression that were calculated outside the tested sampling period were excluded and converted to a T90 value greater than or less than the sampling timeframe (> or <).

Results

Viral inactivation across OC43, MHV, and phi6 generally increased with increasing temperature and relative humidity with phi6 without mucin being readily inactivated across all conditions except for the lowest temperature and relative humidity (Figure 2.1). OC43 was the hardiest of the coronaviruses where with T90 values ranging from 50.9 hours (25 °C, 23% RH) and 2.5 to 4.7 hours (65% RH, both temperatures) (Table 2.1). MHV inactivation data was less complete but followed trends with increasing

die-off with increasing temperature and humidity (Table 2.2). Experiments with MHV at 65% RH were conducted, but inoculated virus seemed to be below the limit of detection before the first time point (2hr) requiring further follow up.

General inactivation trends held true for phi6 with and without added bovine mucin background. Adding bovine mucin matrix preserved phi6 viral viability during persistence experiments where, the T90 at 25 °C and 23% RH was beyond the sampling period (>48 hours) (Table 2.3) Without bovine mucin, phi6 at 25 °C and 23% RH has a T90 of 21 hours (Figure 2.2).

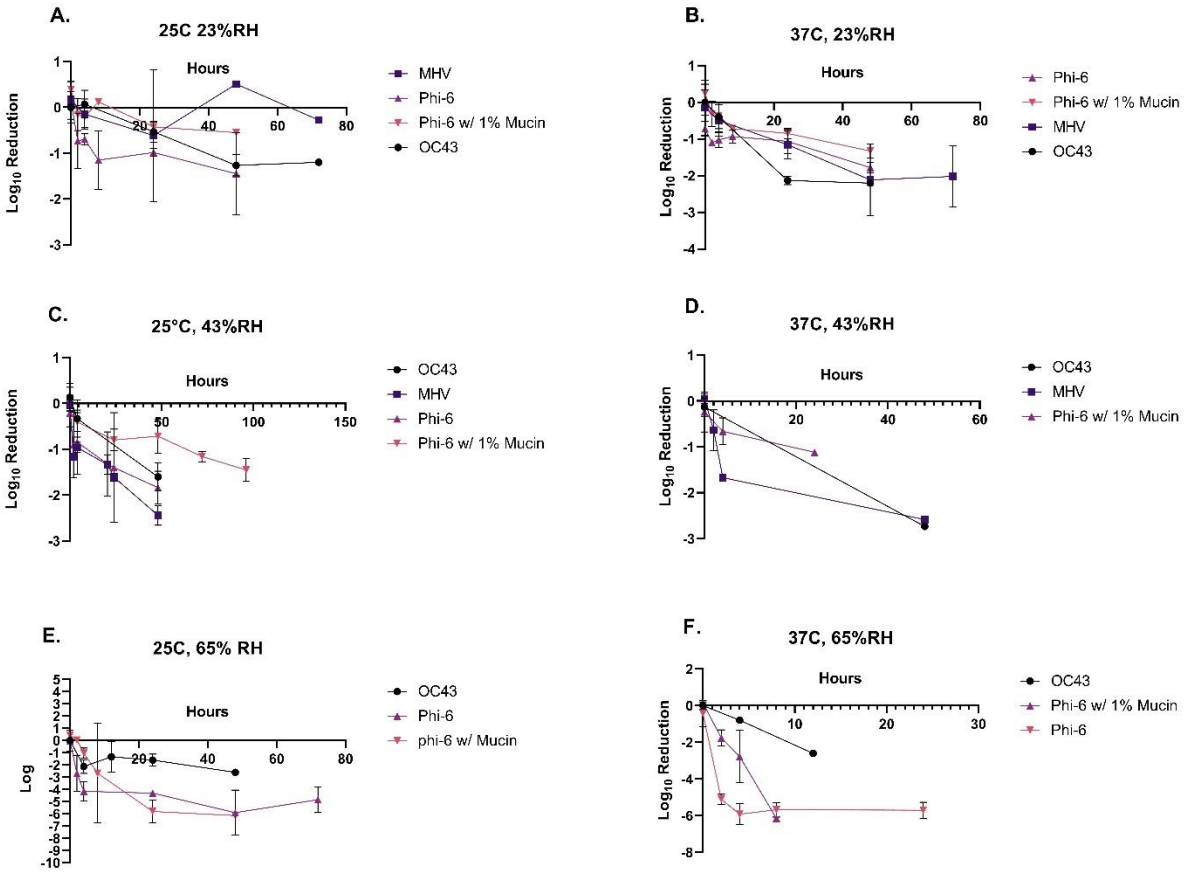

Figure 2.2 **Log10 reduction values longitudinally for HCoV-OC43 (Black), MHV (purple), and phi6 with and without 1% bovine mucin on stainless steel discs.** Each graph represents a different environment combination of relative humidity (%) and temperature. Relative humidity levels tested were 23%, 43% and 65% across 25 °C and 37 °C.

Table 2.1 Estimate T90 values (hrs) for HCoV-OC43 (TCID50). Estimated time in hours it takes for OC43 to decrease by 90% based on tested temperatures (°C) and relative humidity (±8%).

	25 °C	**37 °C**
23% RH	50.9	15.1
43% RH	29.4	16.0
65% RH	2.5	4.7

Table 2.2 Estimate T90 values (hrs) for MHV (PFU). Estimated time in hours it takes for MHV to decrease by 90% based on tested temperatures (°C) and relative humidity (±8%)

	25 °C	**37 °C**
23% RH	No observed die-off[*]	22.8
43% RH	11.4	12.8
65% RH	NA[ŧ]	NA[ŧ]

[*]No observed die-off, T90 calculations could not be estimated due longitudinal data
[ŧ]NA, Data not available

Table 2.3 Estimate T90 values (hrs) for phi6 (PFU). Estimated time in hours it takes for phi6 to decrease by 90% with and without 1% mucin added background based on tested temperatures (°C) and relative humidity (±8%).

	Unaltered Background		**1% Mucin**	
	25 °C	**37 °C**	**25 °C**	**37 °C**
23% RH	21	8	>48	31.9
43% RH	12.6	NA	51.3	20.1
65% RH	<2	<2	3.3	<2

Discussion

Coronaviruses MHV and HCoV-OC43 and bacteriophage phi6 followed observed die-off trends in relation to increasing temperature and relative humidity (Figure 2.2). These trends fall in line with previous work looking at the relationship between temperature and relative humidity (Guillier, Martin-Latil et al. 2020), but further work is needed to better understand this multi-variable relationship in this research. Based on estimated T90 values for all our tested viruses, it appears that they die-off more quickly than two published SARS-CoV-2 survival values (Chin, Chu et al. 2020, Morris, Yinda et al. 2020), this suggests that these surrogates are not conservative markers for SARS-CoV-2 or that there are underlying variables that must be addressed. Previous surface survival studies using phi6 also suggest that

this phage is not an appropriate surrogate for SARS-CoV-2 work in the environment due to it being more labile than coronaviruses (Aquino de Carvalho, Stachler et al. 2017, Whitworth, Mu et al. 2020).

At tested temperatures 25 °C and 37 °C, we found that generally there was increased viral die-off with increasing temperature except for two cases (HCoV-OC43 at 65% RH, and MHV at 43% RH) where differences in the T90 were the most rapid but only varied by a few hours (Table 2.1 & Table 2.2). Tested relative humidity (23, 43, and 65%) showed more substantial changes than temperature, where increasing relative humidity led to increased viral die-off in all experiments. NIOSH recommendations for ambient conditions suggest that occupied buildings should remain between 20-23 °C and maintain a relative humidity between 30-60% (NIOSH, 2015) for occupancy comfort and to prevent fungal and mold growth. This work used temperature that would be much higher than those found indoors (25 and 37 °C) due to incubator capacity to maintain consistent temperatures in relation to our ambient laboratory environment. However, we were able to use a wide range of comfortable relative humidity markers (23 %, 43%, and 65%) that would be appropriate for understanding how tested viruses interact in conditions found in most buildings. With implications for the built environment, ambient conditions including temperature, relative humidity, air exchange, and HVAC design have all been cited as mitigation for both surface and aerosol transmission routes (Dietz, Horve Patrick et al. 2020).

In our study phi6 had the greatest decreases in viral viability even though it also had the largest initial viral load (~10^7) compared to MHV and HCoV-OC43. Phi6 die-off was especially steep at highest tested relative humidity (63%) where it lost >6 $\log_{10}$ reduction values in less than 2 hours (Figure 2.2E & F). This was extraordinary considering HCoV-OC43 was quantifiable and was reduced by 90% after 2.5 and 4.7 hours in the same conditions (Table 2.1). Environmental studies looking at initial viral load as a factor has found that this improves viral aggregation increasing viral survival and resistance to disinfection (Gerba and Betancourt 2017, Bangiyev, Chudaev et al. 2021), suggesting that the high initial phi6 load should be *improving* its survival compared with our other tested viruses and that its survival would be even lower if loaded at the same concentration as HCoV-OC43 or MHV.

Background matrices of our tested viruses ranged depending on the concentration method, growth media, or dilution method of our viruses. HCoV-OC43 virus was concentrated using a PEG and NaCl concentration method (Philo, Keim et al. 2021) and then the pellet was resuspended in phosphate buffer solution. MHV stocks were prepared only by clarifying virus and cell lysates via centrifugation and filtering with cell media, sera, and antibiotics. Phi6 stocks were grown in a bacterial media broth and then clarified by centrifugation and filtering much like MHV but then was also diluted 10-fold in PBS with or without mucin. Differences in stock and loading preparations means they also had different background matrices when subjected to variable environments. Testing phi6 against two separate matrix backgrounds (PBS alone or with 1% bovine mucin) yielded dramatic differences in survival, with bovine mucin preserving phi6 and almost tripling many of the T90 values across the same conditions (Table 2.3). Background matrix is well known for affecting viral survival by directly protecting viruses from environmental insults (UV light, temperature), preventing excess desiccation, providing oxidant demand, and/or by enhancing viral aggregation to substrates or other virions (Gerba and Betancourt 2017, Lin, Schulte et al. 2020). Background matrices added experimentally all have preserving effects whether it be Ebola virus with blood (Schuit, Miller et al. 2016), influenza virus H1N1 with mucin and sera (Coulliette, Perry et al. 2013), and human rhinovirus in tryptose, mucin or nasal discharge (Sattar, Karim et al. 1987). These studies fall in line with phi6 results from this study but none of these represent actual respiratory fluids which are argued to be hostile viral environments due to the presence of immune cells, protective enzymes, and antiviral antibodies (Eccles 2020).

Limitations of this work include method for estimating T90 values which was done using simple linear regression where interactions between temperature, relative humidity, and background were not captured and each variable was treated separately. From previous work, environmental conditions involved in viral survival do not operate independently and there is often interactions between different conditions when tested in different conditions (Dean, Wissler et al. 2020). Interactions between relative humidity and temperature have been found in individual studies looking at SARS-CoV-2 (Morris, Yinda et al. 2020), animal coronaviruses (Casanova Lisa, Jeon et al. 2010) or a suite of different coronaviruses

through meta-analysis work (Guillier, Martin-Latil et al. 2020). Additional work is planned to apply a suite of different die-off models that utilize a most-likelihood estimation and multi-variable approach to better understand the contribution of environmental conditions and better understand viral inactivation on surfaces. Other limitations that need to be addressed are recovery efficiencies for viruses on surfaces to investigate whether one virus experienced less recovery than others and if that would affect results presented in this report.

This research is timely because it helped to address several novel coronavirus pandemic questions related to viral survival on surfaces using three commonly used SARS-CoV-2 surrogate. Fomite transmission is inherently difficult to study because other transmission routes (direct, droplet, aerosols) are difficult to rule out in cases of suspected outbreaks (National Center for and Respiratory Diseases 2020). Environmental conditions such as initial viral load, temperature, relative humidity, and background matrix are known to have impacts on the survival of viruses on surfaces and were further confirmed in this research. Future research should include the addition of other animal coronaviruses that are also being used as SARS-CoV-2 surrogates and to determine which surrogate is an appropriate virus for disinfection.

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3. Direct comparison of the efficacy of hypohalous acids (HOCl & HOBr) in the inactivation of MS2 Bacteriophage as an Enteric Viral Surrogate

Introduction

Healthcare associated infections occur in 1 in 10 patients in high income countries and almost 1 in 4 patients in low- and middle-income countries causing hundreds of millions of excess infections globally (Pittet, Allegranzi et al. 2008). A substantial portion of these are preventable infections where improved environmental and hand hygiene measures could reduce fomite transmission of pathogens from hands and medical devices. Among the most effective interventions cited in preventing environmentally transmitted infections is through chemical disinfection of surfaces and hands to reduce pathogens that are fastidious survivors in the environment, resist standard disinfection protocols, and/or cause high consequence diseases (Terpstra, van den Blink et al. 2007). Disinfectant efficacy of chemical disinfectants can differ based on the target pathogen, organic loading, contact time, concentration, and chemical composition. Determining the application and efficacy of chemical disinfectants must be evaluated individually.

Hypochlorous acid (HOCl) is a weak acid that is generated electrochemically to produce chlorine at relevant levels for surface disinfection. HOCl has demonstrated broad microbial disinfection and deodorizing capabilities for surface and aerosol disinfection including disinfection of norovirus (Park, Boston et al. 2007), influenza (Hakim, Thammakarn et al. 2015), and enteric viral surrogates (Dennler-Church, Butz et al. 2020). As a disinfectant, HOBr can be easily generated through the addition of NaBr to HOCl at molar equivalents (Kumar and Margerum 1987) and also has demonstrated antimicrobial properties (Hoehn 1976).

Speciation states of both HOBr and HOCl is dependent on the pH of the environment, where increasing pH leads to higher concentration of $^{-}$OCl and $^{-}$OBr changing the oxidation-reduction potential of the product and disinfection capacity. To date, most disinfection studies have examined HOCl and

HOBr separately or utilized products with a mixture of ionization species (*i.e.* HOCl and $^{-}$OCl), making it difficult to directly compare between studies and to parse out the disinfection efficacy that HOCl and HOBr provide alone. Prior research has found that HOBr is a more effective disinfectant than HOCl on poliovirus (Keswick, Fujioka et al. 1982) and bacteria (Gottardi, Klotz et al. 2014) but there are limited numbers of direct comparisons of HOCl and HOBr disinfection using pure solutions of HOCl and HOBr.

To elucidate the potential advantages of either HOCl or HOBr in surface disinfection, we performed a direct comparison of these two hypohalous acid against MS2 bacteriophage (MS2). Additionally, HOBr and HOCl were characterized via spectrophotometric and spectroscopic methods to determine the stability and speciation state of the hypohalous acid. HOCl and HOBr showed differences in their reactivity against the tested viral surrogate.

Materials & Methods

Reagents for the iodometric titrations were purchased from Hach (Dissolved Oxygen 3 Powder Pillows, Potassium Iodide Powder Pillows, Sodium Thiosulfate Digital Titrator Cartridge, 0.113 N, and starch indicator solution. Sodium chlorate ($NaClO_3$) and the platinum standard solution (49763) were purchased from Sigma Aldrich. Sodium chloride (NaCl), Sodium bromide (NaBr) and sodium phosphate monobasic monohydrate (NaH_2PO_4) were purchased from Fisher Scientific. Water from MilliQ water purification system was used for all experiments. HOCl in 2% NaCl was provided by Briotech Inc. in amber glass. HOCl samples were adjusted to the desired pH using 1M NaOH and stored in amber glass vials. An Accumet pH meter was used to obtain pH measurements. The ORP measurements were collected using the ORP sensor (ORP-BTA) and analyzed using the LabQuest®2 (LABQ2) apparatus from Vernier software and technology.

***Iodometric Titration*.** The active chlorine concentrations in the HOCl and HOBr solutions was determined following previously described methods and HACH reagents (Hughson, Race et al. 2016).

Heated samples were kept at 52 °C and 70 °C in water baths and were allowed to equilibrate to room temperature before iodometric analysis. Each sample was titrated a minimum of three times.

Conversion of HOCl to HOBr. The conversion of HOCl to HOBr was achieved by the addition of NaBr (1.1 molar eq) to HOCl at pH 4. The conversion was monitored by UV-VIS (BioMate 3S). The absorbance of HOCl at 236 nm shifted to 260 nm (Kumar and Margerum 1987).

Characterization of HOCl and HOBr by UV-Visible Spectroscopy. Ultraviolet Visible spectra were collected using a BioMate 3S UV-Visible Spectrophotometer. The instrument was calibrated using Nanopure water. Test solutions were loaded in a 1 cm quartz cuvette (Sterna Cells). The absorbance was measured from 190–400 nm. The absorbance of HOCl solutions were measured at pH 3.7, 7.0, and 11.8. The pH was adjusted by the addition of 1M NaOH. The chemical integrity of HOBr was monitored at 260nm (Kumar and Margerum 1987).

Raman Spectroscopy. Spectra were obtained using a Renishaw InVis Raman microscope. Spectra were observed using an excitation wavelength of 785 nm for undiluted HOCl and HOBr respectively, in 1 mL quartz cuvettes. HOBr was prepared as previously outlined prior to analysis. The acquisition time for each scan was 20 s, and 100 acquisitions were accumulated with a spectral window from 400 nm – 1000 nm. A 2 % NaCl (W/V) blank solution was scanned using the same method and subtracted from the test sample data.

Inductively coupled plasma optical emission spectrometry (ICP-OES). Trace platinum analysis was performed on Perkin-Elmer Optima 8300 Inductively Coupled Plasma-Optical Emission Spectrophotometer. A calibration curve of platinum was generated from 1-20 ppb. Acid digestion was carried out on Briotech Inc. HOCl with 1:3 HNO_3: HCl, which was tested at various concentrations using ICP-OES. All standards and samples were tested five times.

Surrogate virus and host. MS2 bacteriophage (ATCC 15597-B1) was prepared by confluent lysis on *Escherichia coli* F-amp (ATCC 70081) in 15 ml soft agar (0.25% Bactoagar, 0.7% NaCl) poured onto a nutrient agar petri dish and incubated at 37 °C overnight. Propagated MS2 was further purified via organic solvent extraction using Vertrel XF (Dupont) by vigorously mixing scraped top layer of soft agar and equal volumes of Vertrel XF followed by centrifugation at 3500 x g at 4 °C for 15 minutes. Centrifuged supernatant was collected and stored in 1 ml aliquots at -80 °C. All MS2 stocks and samples were quantified by 10-fold serial dilutions and plating using the Double Agar Layer method as previously described (Adams 1959).

HOCl and HOBr Disinfection Comparison of a Viral Surrogate. MS2 stocks used in disinfection trials were diluted in Nanopure water (pH= 4.8) and filtered to reduce aggregation using a 0.22um PVDF filter. Disinfection trials were carried out by taking 960 µM HOCl and HOBr and serially diluting (2-fold) in Nanopure water (pH= 4.8). 900ul of 0, 60, 120, 240, and 480 µM HOCl or HOBr was exposed to ~10^7 of MS2 in demand free glassware for 30 or 60 seconds prior to quenching using phosphate buffer solution with 1% sodium thiosulfate. Quenched disinfection samples were then quantified by plating relevant dilutions using the double agar layer method (Adams 1959). Pathogen volume in disinfection trials was less than 90% of total reaction volume to reduce influence of chlorine demand from pathogen background and conducted in demand free glassware. All pathogens were tested with HOCl and HOBr at listed concentrations in at least two experiments with 3-5 replicates per experiment. HOBr was freshly prepared and used within one hour of preparation for each experiment to ensure targeted speciation. Demand free glassware was prepared by soaking clean glassware in 5-10% sodium hypochlorite solution for at least one hour. Soaked glassware was rinsed in nanopure water 2-3 times and allowed to air dry prior to use.

HOCl and HOBr Surface disinfection of MS2 on Stainless Steel Surfaces. Sterilized stainless steel coupons were seeded with ~10^7 diluted and filtered MS2 bacteriophage and allowed to dry for 1 hour in a

type II BSC. Seeded coupons were then submerged in 0, 95, 190, 475, 950, or 1400 µM HOCl or HOBr for a contact time of 5 minutes prior to quenching using PBS with 1% sodium thiosulfate. Samples were then processed and quantified for viable MS2 by plating relevant dilutions using the Double Agar Layer method (Adams 1959). Experiments had 5-10 replicates in each experiment with two independent experiments. Sample concentrations were normalized by taking the $\log_{10}$ of each sample. The average of normalized non-treated samples was used to determine $\log_{10}$ reduction values for each treatment. Unpaired student t-tests with Welch correction ($\alpha = 0.05$) were used to compare $\log_{10}$ normalized HOCl and HOBr disinfection at equivalent contact times and concentrations of MS2 bacteriophage in suspension from two independent experiments with three replicates of each treatment variable. All graphs and statistical analyses were made in GraphPad Prism version 9.0.2 (161) (San Diego, CA).

Results

Characterization of HOCl and HOBr Solutions. HOCl solutions showed the expected UV-Visible absorption maxima at 236 nm in samples near pH 4 (Figure 3.1 A & B). A mixture of both HOCl and OCl- was detected by UV-Vis after the pH was adjusted to 7 and complete conversion to OCl- was detected after pH adjustment to 11.8 (Adam, Fabian et al. 1992) The conversion to HOBr occurred immediately after the addition of NaBr with an absorption maxima shift to 260 as expected for HOBr. The maxima shifted further to 329 nm for OBr- after pH adjustment to 11 (Faria, Epstein et al. 1994, Beckwith and Margerum 1997, Liu and Margerum 2001).

Raman spectroscopy was used to further characterize fresh HOCl and freshly prepared HOBr solutions. In HOCl solutions at pH 4 a single peak was detected by Raman spectroscopy at 728 cm^{-1}, corresponding to expected resonance for the Cl-O stretch of HOCl (Figure 3.1 C & D) (Nakagawara, Goto et al. 1998) Raman resonances for the decomposition chlorine chemical species, including Cl2 (540 cm^{-1}), ClO2-1 (799 cm^{-1}), and $ClO3^{-1}$ (934 cm^{-1}) were not detected under the experimental conditions (Yuan, Garver et al. 2004). After conversion to HOBr the Raman resonance shifted to 626 cm-1, as expected (Evans and Lo 1967).

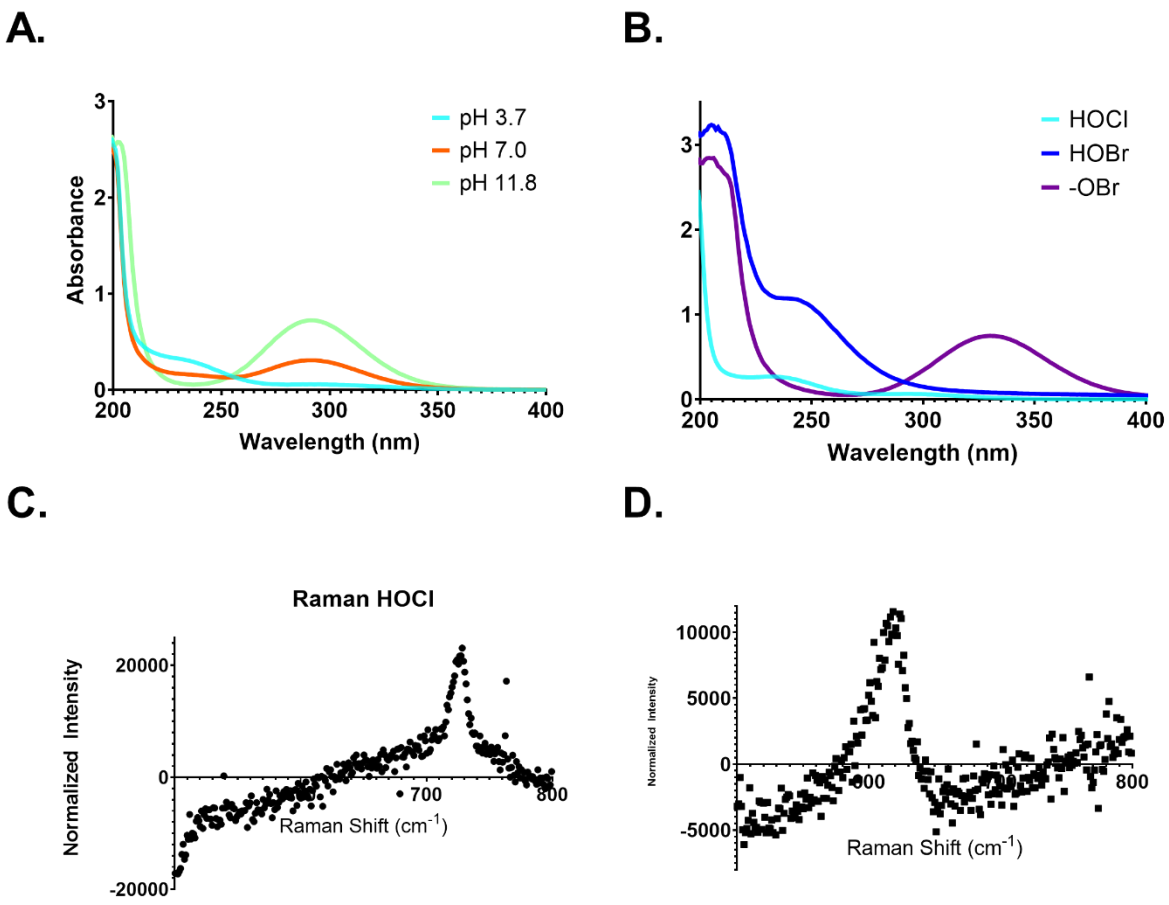

Figure 3.1. **Characterization of HOCl and HOBr by UV-Visible and Raman spectroscopy at 22 °C. A.** UV-Vis spectra of 147 ppm HOCl at pH 3.7, -OCl at pH 11.8 and a mixture of HOCl and -OCl at pH 7.0. **B.** UV-VIS spectra of HOCl (pH 4.0, 3.0 mM), HOBr (pH 6.2, 3.0 mM), and OBr- (pH 11.8, 3.0 mM). **C.** Raman spectra of HOCl (5.3 mM). **D.** Raman spectra of HOBr (1.8 mM).

Stability of HOCl and HOBr Solutions. The concentration of active chlorine, ORP and pH remained relatively constant at 175 ppm, pH 4, and ~1100 mV over >90 days at ambient temperature in glass storage vessels. At elevated temperatures of 52 °C and 70 °C, the titratable chlorine concentrations in HOCl solutions declined as shown in Figure 3.2 after monitoring periods of 86 and 61 days, respectively. No platinum from production electrodes was detected in final HOCl samples by IEP-OES, UV-Visible spectrophotometric data, and Raman spectroscopy indicating the presence of only HOCl in test solutions.

HOCl adjusted to pH 7 and 70 °C showed a rapid decline in active chlorine concentration and pH. The ORP of this solution increased over the testing period.

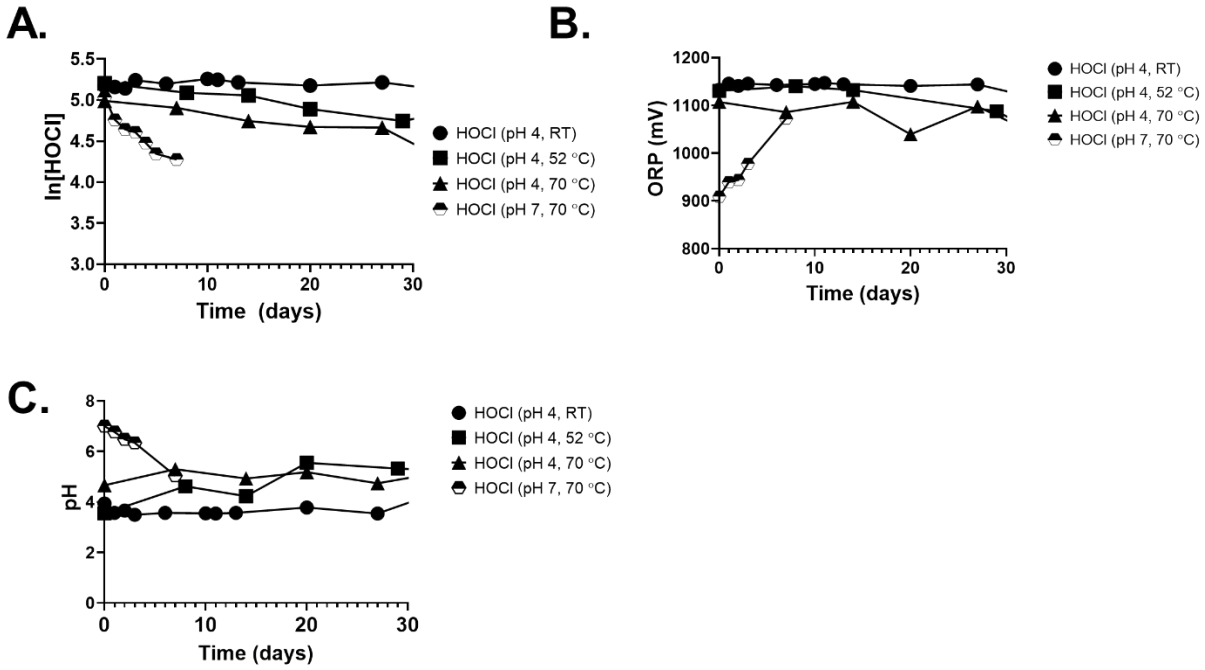

Figure 3.2. **Stability of HOCl.** A. Pseudo first order plot of the temperature dependence of HOCl at 22 °C, $pH_{initial}$ = 4; 52 °C, $pH_{initial}$ = 4; 70 °C, $pH_{initial}$ = 4; and 70 °C, $pH_{initial}$ = 7. B. ORP measurements of HOCl solutions at 22 °C, $pH_{initial}$ = 4; 52 °C, $pH_{initial}$ = 4; 70 °C, $pH_{initial}$ = 4; and 70 °C, $pH_{initial}$ = 7. C. pH values of HOCl solutions at 22 °C, $pH_{initial}$ = 4; 52 °C, $pH_{initial}$ = 4; 70 °C, $pH_{initial}$ = 4; and 70 °C, $pH_{initial}$ = 7.

In contrast, HOBr solutions rapidly decayed at ambient temperature (Figure 3.3A). Changes in absorbance by UV-Visible spectroscopy were detectable within minutes (Figure 3.3B) and over the full testing period (9 days) decomposition led to generation of other active bromine species; however, active Br concentrations detected by iodometric titration remained constant (Beckwith and Margerum 1997).

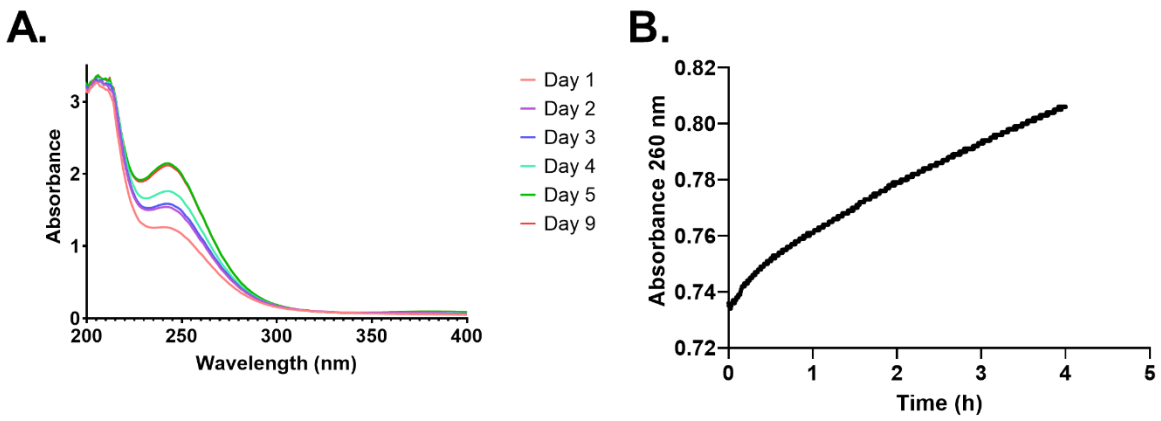

Figure 3.3. **Stability of HOBr. B.** UV-Visible spectroscopy for HOBr at 22 °C over 9 days; **C**. UV-Visible spectra measured at 260 nm of HOBr at 22 °C for 4 h.

Disinfection trials. HOBr and HOCl were both effective disinfectants in suspension against MS2 achieving > 4 log10 reductions required of disinfectants with virucidal claims (US EPA, OCSPP 810.2200) within 30 and 60 seconds at the two highest concentrations of HOCl and HOBr of 240 or 480 µM (Figure 3.4 A &B). At 30 seconds of contact time 4.26 (±0.76 95% CI) and 3.44 (0.36 95% CI) $\log_{10}$ reductions, respectively, were achieved with 240 µM of HOBr and HOCl. At 60 seconds of contact time and 240 µM, 5.6 (1.37 95% CI) and 3.9 (0.76 95% CI) $\log_{10}$ reductions were observed for HOBr and HOCl, respectively. HOBr had greater variability (Table **3.1**, 95% CI) within treatment than HOCl but consistently outperformed HOCl during disinfection trials (Table **3.1**). The enhanced disinfection capability of HOBr compared with HOCl was statistically significant with 30 seconds of contact time at 240 and 480 µM (p-values= 0.041 and 0.0085) and 60 seconds of contact time at 60, 120, and 240 µM (p-values= 0.046, 0.03, 0.02). For 60 seconds of contact time HOCl and HOBr both resulted in inactivation beyond the limit of detection (LOD= 7.3-7.4) and samples were indistinguishable from each other (Table **3.1**).

On surfaces, HOCl and HOBr were also effective disinfectants at 5 minutes contact time achieving >4 log10 reduction values at concentrations at and above 950 µM of HOCl and 475 µM of HOBr (Figure 3.5). At the lowest concentration (95 µM) HOCl did not result in any disinfection (0.0

LRV ± 0.1 95% CI) with HOBr at 95 µM having minor disinfection with 0.8± 0.5 95% CI (Figure 3.5). Beyond 95 µM, HOCl and HOBr followed consistent linear log_{10} decreases with increasing disinfection concentration (Figure 3.5). Similar to suspension tests (Figure 4) HOBr experienced more variability and inactivation of MS2 than HOCl at each tested concentration (Figure 3.5). At 1400 µM, both HOCl and HOBr samples were below limit of detection with ≥ 6.7 log_{10} reductions.

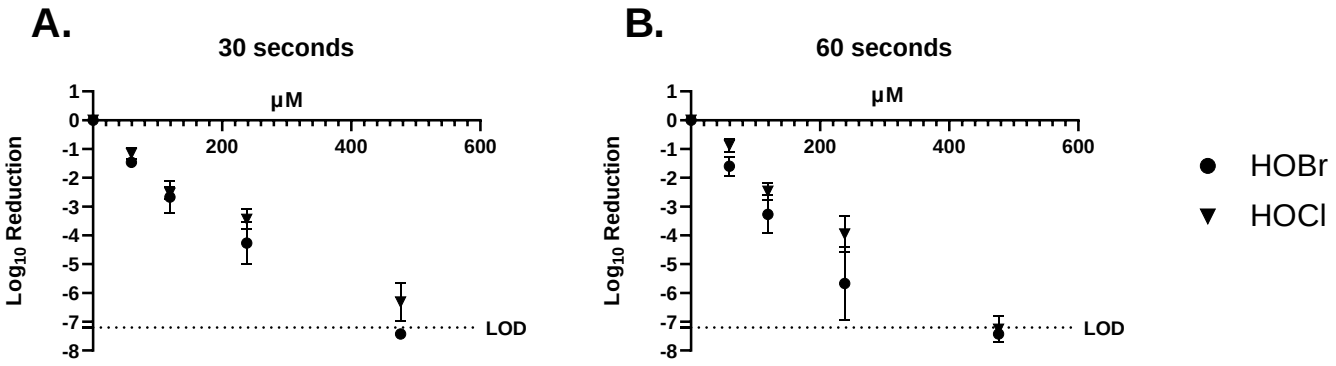

Figure 3.4. **MS2 bacteriophage disinfection in suspension.** Mean Log10 reduction values (±SD) for the disinfection of MS2 bacteriophage using HOCl and HOBr at A. 30 seconds contact time and B. 60 seconds contact time in suspension.

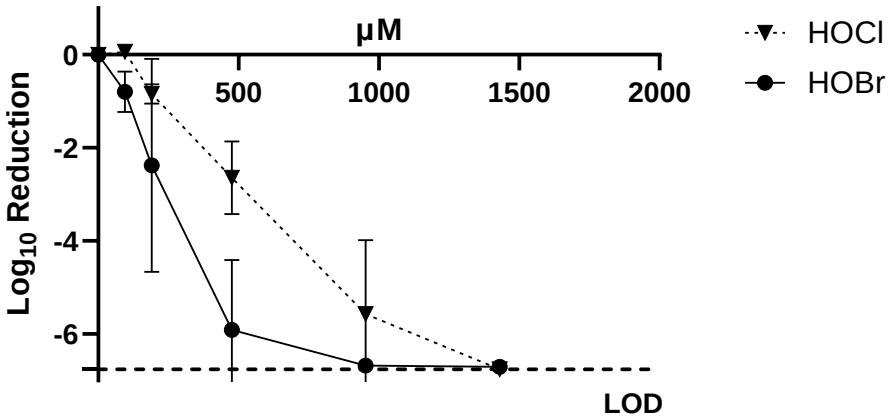

Figure 3.5. **MS2 bacteriophage HOCl and HOBr disinfection on stainless steel**. Mean Log10 Reduction values (±SD) disinfection of dried MS2 bacteriophage on stainless steel coupons at varied concentrations of HOCl and HOBr after 5 minutes of contact time.

Table 3.1 HOCl and HOBr MS2 bacteriophage disinfection in suspension comparison at 30 seconds and 60 seconds of contact using concentrations of 60, 120, 240, and 480 µM HOCl and HOBr. Data is presented as summary statistics with Log_{10} normalized data with unpaired student t-tests with Welch correction and $\alpha = 0.05$.

Treatment	Mean Log_{10} ± 95% CI	σ_x	N	LRV± 95% CI	Treatment	Mean Log_{10} ± 95% CI	σ_x	N	LRV± 95% CI	p-value	
Reference	8.1 ± 0.1	0.15	12								
HOCl: 30 sec					**HOBr: 30 sec**						
60 µM	6.9 ± 0.2	0.19	3	1.3 ± 0.2	**60 µM**	6.6 ± 0.2	0.14	3	1.5 ± 0.2	0.094	ns
120 µM	5.6 ± 0.2	0.19	6	2.5 ± 0.2	**120 µM**	5.4 ± 0.4	0.44	5	2.7 ± 0.4	0.630	ns
240 µM	4.6 ± 0.3	0.38	6	3.5 ± 0.3	**240 µM**	3.9 ± 0.7	0.83	6	4.3 ± 0.7	0.041	*
480 µM	1.8 ± 0.5	0.63	6	6.3 ± 0.5	**480 µM**	< 0.7 ± 0.0	0.00	6	7.4 ± 0.1	0.008	**
HOCl: 60 sec					**HOBr: 60 sec**						
60 µM	7.2 ± 0.3	0.22	3	0.9 ± 0.3	**60 µM**	6.4 ± 0.4	0.34	3	1.7 ± 0.4	0.046	*
120 µM	5.6 ± 0.2	0.22	6	2.5 ± 0.2	**120 µM**	4.9 ± 0.5	0.65	6	3.3 ± 0.5	0.030	*
240 µM	4.2 ± 0.6	0.69	5	3.9 ± 0.6	**240 µM**	2.4 ± 1.1	1.40	6	5.7 ± 1.1	0.020	*
480 µM	< 0.9 ± 0.3	0.39	6	>7.3 ± 0.3	**480 µM**	< 0.7 ± 0.0	0.00	6	>7.4 ± 0.1	0.437	ns

Unpaired student t-tests with Welch correction and α= 0.05 were used to compare HOCl and HOBr disinfection at equivalent contact times and concentrations of MS2 bacteriophage in suspension. Results included are from two independent experiments.
p < 0.05 = *
p < 0.001 = **
not significant = ns

Discussion

Given the previous work demonstrating the antimicrobial properties of HOCl and HOBr, we expected and observed that HOCl and HOBr were effective disinfectants on MS2 at room temperature, two different contact times, and several concentrations (Park, Boston et al. 2007, Gottardi, Klotz et al. 2014, Dennler-Church, Butz et al. 2020). Comparisons between the two disinfectants resulted in higher efficacy of HOBr compared to HOCl at almost every concentration and contact time tested (Figure 3.4Figure 3.5). Based solely on ORP, we expected and observed HOBr to have a stronger disinfection capacity across all but a few conditions which is supported by previous findings using poliovirus (Keswick, Fujioka et al. 1982) and bacterial species *E.coli* and *S. aureus* (Gottardi, Klotz et al. 2014). Added benefits of HOBr in disinfection capabilities for non-enveloped viruses on surfaces were further

highlighted by additional testing of HOBr and HOCl with dried MS2 on stainless steel coupons (Figure 3.5) demonstrating practical use of HOBr for enhanced surface disinfection.

In surface experiments, HOBr provided more disinfection to dried MS2 on stainless steel discs compared to HOCl, where HOBr provided 5.9 (± 1.1 95% CI) log_{10} reductions at 480 µM HOBr while HOCl provided 2.6 (±0.6 95% CI) Log_{10} reductions in the same environmental conditions (Figure 3.5). HOBr had more variability in these experiments but consistently provided greater inactivation of MS2 (Figure 3.5). In foodservice settings where incidences of diarrhea or vomit containing norovirus from customers or employees, insufficient cleaning and contaminated surfaces have led to large outbreaks with high attack rates from norovirus transmitted via fomites (de Wit, Widdowson et al. 2007, Repp and Keene 2012). One area of further research would be the impact of environmental oxidant demand of fecal and vomit on HOBr vs HOCl surface disinfection. One previous comparison study has shown that the enhanced disinfection of HOBr can be negated with more complex background matrices (Gottardi, Klotz et al. 2014), where bromine performs better with less protein loading such as skin and pre-cleaned surfaces. This research used diluted 1/10 and filtered MS2 stocks, making them close to but not zero demand free experiments where added disinfection capacity of HOBr was comparable to previous reports (Gottardi, Klotz et al. 2014). Additional work on different load scenarios is needed to better elucidate the impact background matrix would have on HOBr surface disinfection. Fortunately, the Gottardi study also demonstrated that while enhanced disinfection from HOBr was negated, HOBr and HOCl still had equal disinfection capabilities regardless of background matrix. In practice, it is important that there was no added penalty based on disinfection choice in these settings.

Studies using MS2 as an enteric virus model have found that hypochlorous acid disinfectants act by modifying capsid proteins before structural dissolution and nucleic acids degradation (Wigginton, Pecson et al. 2012, Sigstam, Gannon et al. 2013). While this work did not look specifically at inactivation mechanisms, HOCl and HOBr readily react with protein residues with *in vitro* HOBr being more reactive than HOCl, and HOCl being more reactive than amine conjugated chlorine and bromine species (Gottardi, Klotz et al. 2014). Reaction rate constants of HOBr compared to HOCl have found that overall HOBr

reacts more quickly with amino acids and proteins with the exception of cysteine and methionine where HOCl is more reactive (Pattison and Davies 2004). More complex biological molecules also found that HOBr can oxidize molecules that are otherwise unreactive in the presence of HOCl (Ximenes, Morgon et al. 2015). HOBr readily reacts with tyrosines, while HOCl was found to react more readily with methionines in isolated MS2 capsid proteins with 3D geometric structure of the virion capsid driving inactivation rates of HOCl and HOBr (Choe, Richards et al. 2015). Methionine-108 in the MS2 capsid was been identified as an HOCl oxidized residue (Bastin, Loison et al. 2020) but there is less available information on HOBr modifications on MS2 capsid proteins. When it comes to precise disinfection mechanisms it is clear that the 3D geometry of the MS2 capsid proteins and genomic RNA as well as the amino acid composition (Golmohammadi, Valegård et al. 1993, Ni, Syed et al. 1995, Bastin, Loison et al. 2020) are important factors during hypohalous disinfection that require further work.

Characterization of HOBr and HOCl products showed that protonated HOCl was stable for the long periods at pH=4 with increasing pH resulted in resulted in faster degradation and a less stable disinfectant (Figure 3.3 & Figure 3.2). As a result of the stability and characterization data in this research, all disinfection studies were conducted at a lower pH (pH ≈ 5) than previously described studies that use HOCl at or near pH levels appropriate for drinking water disinfection systems pH= 6.5-8.5 (US EPA: Safe Drinking Water Act) to ensure that HOCl and HOBr solutions were pure. Storage at lower pH would also increase shelf-life of the product allowing for a longer lasting product for consumers (Figure 2A, B, C). Similar stability and characterization experiments (Figure 3B) showed that HOBr is less stable than HOCl (Figure 3.3A) and speciation of HOBr quickly degraded over the course of a few hours indicating that surface disinfection of HOBr must be produced on-site and used within a certain window.

While HOCl and HOBr are effective disinfectants, there are concerns for exposure to chlorinated and brominated by-products some of which are known carcinogens (US EPA: Safe Drinking Water Act), occupational respiratory irritants (Florentin, Hautemanière et al. 2011), and have also been found as *in vivo* inflammatory biomarkers (Kettle, Albrett et al. 2014). In their pure forms, HOCl and HOBr are well tolerated by mucus membranes and around the eyes (Stroman, Mintun et al. 2017) compared with

household bleach because of high hypochlorite concentrations and pH, making it preferable for cleaning and disinfection purposes. In this study we found that due to its high reactivity, HOBr changed within a matter of hours indicating that other bromine species were likely forming changing disinfectant capabilities and potentially its tolerability and toxicity (Ximenes, Morgon et al. 2015). While no disinfectant is without some sort of cost these considerations must be balanced with other hospital grade disinfectants used to reduce pathogen loads of some spore-forming bacteria and other highly resistant pathogens that often require highly destructive and toxic disinfection and sterilization methods. These include disinfectants such as ethylene oxides, glutaraldehyde, and sodium hydroxide solutions which must be handled by trained individuals often in isolation of others. A better understanding of toxicity of hypohalous acids in comparison to other disinfectants would be necessary to fully understand their potential benefits in these settings.

This study was one of the first to do a direct disinfection comparison of pure solutions of HOCl and HOBr on MS2 bacteriophage. Previous research has demonstrated disinfection efficacy of HOCl and HOBr but few have done direct comparisons with pure solutions of HOCl and HOBr. While HOBr provided enhanced disinfection compared with HOCl (Figure 3.4 & Figure 3.5), our research did not explore the mechanisms behind these differences. Future research on these mechanisms could include identification of protein modifications by HOCl and HOBr on MS2 protein capsids and genomic RNA using SDS-Page and Mass Spectrometry methods. Additional research on the role of 3D geometry of amino acids and secondary structures could also help to develop predictive modelling of oxidative disinfection process across disinfectants with similar oxidative disinfection mechanisms and for other viral pathogens implicated in fomite transmission. Chemical characterization found that HOCl held desired chemical properties at a variety of holding temperatures for over 30 days when kept at low pH. HOBr was readily produced as indicated by absorbance and ramen spectrometry shifts but changed within hours indicating the need for on-site production and immediate use (Figure 3.2 & Figure 3.3). Beneficial implementation of environmental hygiene and surface disinfection using hypohalous acids could mitigate a range of fastidious pathogens where other disinfectants are incomplete in their ability to inactivate

biohazards or create a chemical toxicity hazards themselves. While hypohalous acids are well known disinfectants this study was one of the first side-by-side comparisons that controlled for chemical speciation and did a full characterization on stability in storage. Future research will be to expand the suite of tested pathogens to better understand the extent of enhancement HOBr provided by investigating disinfection mechanisms of HOBr through advanced protein analysis techniques.

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4. Chemical Disinfection to Remove Viral Pathogens and Surrogates from Research Animal Wastewater to Address Occupational and Environmental Concerns

Introduction

Recombinant viruses and viral vectors are valuable biomedical tools used in research to deliver and/or integrate nucleic acids for gene therapy, cell genome modification, or vaccine development. Viral vectors come in the forms of adenoviruses, retroviruses, herpesviruses, poxviruses, and Sendai viruses that are modified to deliver or integrate recombinant nucleic acid with or without an accompanying virus (helper virus) into host cells. Viral vectors are a highly efficient tool for cellular manipulation and has been widely used in biomedical research, but development of viral vectors requires a number of biosafety measures to ensure that viral vectors with toxigenic or oncogenic nucleic acids end up in unsuspecting hosts or the environment (Collins, Reuter et al. 2017). Shedding of recombinant viruses are a concern during research as they might be exposed occupationally to animal caretakers or to other unsuspecting hosts via the environment through feces and wastewater conveyance. Previous studies looking at recombinant viruses in both human and research animal trials have found that inoculated recombinant viruses can be shed for as long and in concentrations comparable to infection with a wild-type virus (Murphy Brian, Markoff Lewis et al. 1978, Natuk, Lubeck et al. 1993). In contrast, viral vectors systems have been in use for decades, but there is a paucity of shedding data in the literature (Schenk-Braat, van Mierlo et al. 2007) in the form of clinical trials, pharmaco-kinetic studies or from replicant conversions. Of the studies that have been conducted, there was no evidence of shedding or shedding at low rates (Schenk-Braat, van Mierlo et al. 2007, Cesani, Plati et al. 2015, Farraha, Barry et al. 2019). Development of recombinant viruses and clinical grade viral vectors is an arduous process (Collins, Reuter et al. 2017) but more information potential occupational and environmental hazards via shedding must be done.

Non-human primate waste containing recombinant viruses and viral vectors can be both an occupational and environmental concern for non-human primate research facilities conducting biomedical

research. Until recently, Washington National Primate Research Center (WaNPRC) (Seattle, WA) relied on researchers to carry and manually empty non-human primate habitat catchments containing animal excreta, loose bedding, and food into biohazardous waste containers prior to autoclaving or treatment with sodium hypochlorite; creating major ergonomic and direct contact hazards with biohazards. To solve ergonomic hazards, a water-based wash system was implemented where habitats are sprayed down and feces, bedding, and wash water is collected into a trough catchment where it could be pre-treated with a chemical disinfectant prior to disposal. While some of these hazards are mitigated through this system, WaNPRC is subject to a suite of regulatory bodies ranging from institutional animal welfare and environmental safety requirements, US National Institute of Health (NIH) animal welfare, and US NIH recombinant nucleic acid guidelines requiring testing, containment, and disposal of any replicant competent viral vectors (RCV) and viral pathogens in animal studies at a biosafety level 2 or higher (NIH Office of Science Policy: Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules, 2019). Biosafety practices dictate how viral vectors are handled and developed where fecal waste from inoculated research animals need to be treated as infected/biohazardous to ensure containment of recombinant viruses and viral vectors.

While many treatment options for biohazardous wastewater and viruses exist, chemical disinfection was easily adoptable in this research facility where catchments of heterogenous wastewater could be treated, and to prevent the loss of contaminant. Survival in environment of viral vectors and viral pathogens is largely dependent on the viral structure, genome type, enzymes, and membrane envelope where these differences can infer disinfection resistance. Therefore, the objective of this study was to examine the use of two off-label chemical disinfectants, one quaternary ammonium disinfectant (QAD) and (KOH-D) potassium hydroxide detergent, to inactivate viral pathogens and surrogates in wastewater from a primate research facility. To do this we looked at the neutralization of Simian retrovirus-2 (SRV-2), Human Adenovirus-2 (HAdV-2), Human Coronavirus-OC43 (HCoV-OC43), *Pseudomonas* bacteriophage phi6 (phi6), and MS2 bacteriophage (MS2) seeded into wastewater from non-human primates to represent viruses that are commonly used as viral vectors in biomedical research.

Additionally, wastewater characterization including bacterial loads, total solids, and viral partitioning was conducted to better understand the utility of liquid disinfection to mitigate occupational biohazards.

Materials & Methods

Viruses and host organisms. A549 cells were grown in monolayers using F- 12K media (ATCC 30-2004) and 10% FBS (ATCC 30-2020). Human Adenovirus-2 (HAdV-2) are grown in monolayers A549 cells (ATCC CCL-185) for 7-10 days until CPE was visible. Viral stocks were prepared from infected monolayer by freezing and thawing three times, followed by organic solvent extraction using Vertrel XF (Dupont). HAdV-2 infectivity was quantified using a 50% cell culture infectious dose dilution endpoint in A549 cells to yield the TCID50 ml^{-1} 7-10 days post infection. A549 cells infected with HAdV-2 were overlaid with F-12K media, gentamycin, kanamycin, nystatin, and 2% FBS. TCID50 values were calculated using the Spearman and Karber algorithm (Hierholzer and Killington 1996). HAdV-2 qPCR assay was based on previously described primers and probes (Heim, Ebnet et al. 2003) and run using Universal Probes kit (BioRad) on a CXF96 Touch Real-Time PCR detection System (Biorad).

MS2 (ATCC 15597-B1) was prepared by confluent lysis on *Escherichia coli F-amp* (ATCC 70081) followed by organic solvent extraction using Vertrel XF. Infectious MS2 was enumerated as previously described double agar layer method on *E. coli F-amp* host (Adams 1959). Using 100 µL aliquots of relevant dilutions in phosphate buffer solution pH=7.4. Plaques forming units mL^{-1} were quantified after incubation at 37°C for 18 hours. Counts over 300 were excluded as too numerous to count.

Samples containing phi6 bacteriophage were enumerated and quantified using methods previously described in Chapter 2 of this work using a double agar layer method (Adams 1959).

HCoV-OC43 stocks were prepared and quantified according to previously described methods (Philo, Keim et al. 2021). All culture-based samples were quantified using the same TCID50 method as stocks. Primers and probes for HCoV-OC43 RT-qPCR assay was based on previous described primers

and probes (Vijgen, Keyaerts et al. 2005) and run using Universal Probes One-step kit (BioRad) on a CXF96 Touch Real-Time PCR detection System (Biorad).

Simian betaretrovirus-2 (SRV-2) was grown in A459 cells and stocks were prepared according to previous work described in (Kuller-2005).

Table 4.1 Categories of viral pathogens and surrogates used in this study based on family/genus, structure, genome, size, and hosts used in this study

Virus	Family/genus	Structure	Genome	Size	Host
Pseudomonas virus Phi-6	Cystoviridae/*Cystovirus*	Enveloped, double capsid	dsRNA segmented 13.5kb	85 nm	*P. syringae*
Human Coronavirus OC43	Coronaviridae/ *Betacoronavirus*	Enveloped, spherical	+ssRNA, linear, 27-32kb	120 nm	HCT-8 cells
Escherichia virus MS2	Leviviridae/*Levivirus*	Non-enveloped, icosahedral	+ssRNA linear, 3.5kb	26 nm	*E. coli*
Human Adenovirus 2	Adenoviridae/ *Mastadenovirus*	Non-enveloped,	dsDNA, linear, 35-36kb	90 nm	A549 cells
Simian retrovirus 2	Retroviridae/ *Betaretrovirus*	Enveloped, pleomorphic	+ssRNA, linear, 8-10kb	80-100 nm	A549 cells and Raji cells

Wastewater collection and characterization. Fecal wastewater samples were collected from random healthy pigtailed macaques (*M. nemestrina*) during routine care and normal cleaning processes of habitats and collected into 50 mL conical tubes. Subsequent research activities using combined wash water, bedding, and feces had no contact with animals and were exempt from IACUC approval. Animals were all housed in the Washington National Primate Research Center (WaNPRC), which is an AAALAC accredited facility and complies with all regulations of NIH Office of animal welfare and the UW IACUC. All animals are routinely tested for SRV, STLV, SIV, SFV, Herpes B virus, and measles.

Total coliforms and enterococci concentrations in wastewater were determined using Colilert-18 and Enteroalert methods using IDEXX quantitray-2000 according to manufacturer's instructions (IDEXX Laboratories, Inc). Fecal wastewater was then pasteurized at 65° C for three hours to decrease native microbes for experiments and minimize contamination in cell cultures and phage assays. Total solids of pasteurized wastewater was determined by taking 1 ml and drying at 103° C until completely desiccated

and calculating percent solids based on wet and dry weights (US EPA, Method 1684 2001). Fecal wastewater that could not be handled using a serological pipette occurred in one instance (Pool 2: QAD, 10-minute experiments) and was diluted up to 50% in pure water and total solids of diluted wastewater was noted.

Disinfection trials. Wastewater disinfection was conducted using 1400 µl of wastewater, 500 µl of virus inoculum, and appropriate volumes of PBS or cell culture media (<100 ul) was used to make up a 2ml wastewater microcosm. Each wastewater microcosm was inoculated with prepared enveloped or non-enveloped viral diluted in PBS. For enveloped viruses, ~10^4 TCID50 HCoV-OC43 and ~10^8 PFU phi6 were inoculated into each enveloped virus wastewater microcosm. Non-enveloped viruses, ~10^5 TCID50 HAdV-2 and ~10^8 PFU MS2 bacteriophage were inoculated into the non-enveloped viral wastewater microcosms.

Concentrations of 0.4%, 1% and 1.5% final volume of the quaternary ammonium disinfectant (QAD) (Process NPD One-step, Steris) were tested in wastewater microcosms environments for 5-, 10-, and 20-minute contact times. Recommended disinfection concentration for on-label QAD is 0.4% (1:256) for surface disinfection. After the desired contact time, QAD treated samples were quenched using PBS with 0.7% Lecithin, 1% Tween-80, and 0.1% Peptone. Concentrations of 0.7%, 1%, and 2% of the potassium hydroxide detergent (KOH-D) (Clean-in-place 100, Steris) were used to disinfect wastewater for a single contact time of 10 minutes. The recommended working dilution for KOH-D is 0.7% for surface disinfection of industrial clean rooms and animal facilities. After the 10-minute contact time passed, 5M HCL was used to neutralize KOH-D disinfectant to a pH of 7.0 - 7.4. Trials were all conducted between 20-25°C in a biosafety cabinet (Table 4.2).

After disinfection and neutralization, treated and reference samples were centrifuged for 2500g for 10 minutes at 4°C and supernatant was collected for culture analysis and quantification of viable virus. Due to wastewater cytotoxicity and low stock concentrations of HCoV-OC43 were unable to be quantified in disinfection studies but were included to better understand partitioning with follow up experimentation on disinfection using QAD forthcoming.

SRV-2 was only evaluated for KOH-D using culture and RT-qPCR using previously described methods (White, Todd et al.). All disinfection treatments and viruses were done with five replicates in each experiment including reference samples.

Table 4.2 Table of tested disinfectants Process NPD One-Step (QAD) and CIP 100 (KOH-D), active ingredients, tested contact times and concentrations, and viruses used in experiments

Disinfectant	Active ingredients	Contact times	Concentrations	Viruses Tested
Process NPD One-Step (Steris ®)	Quaternary ammonium compounds: • di-C8-10-alkydimethyl, chlorides (5-10%) • Benzyl-C12-16-alkydimethyl, chlorides (5-10%)	5, 10, 20 minutes	0.4%*, 1%, 1.5%	phi6, MS2, HAdV-2
CIP 100 (Steris ®)	Potassium hydroxide (10-30%) Tetrasodium EDTA (1-5%)	10 minutes	0.7%*, 1%, 2%, 4%	MS2, HAdV-2, SRV-2

*Recommended working dilution for surface disinfection

Viral Partitioning. To better understand location of seeded virus in liquid or pellet portions of the heterogenous wastewater sample, untreated reference samples were used and further processed to collect the spiked homogenate, supernatant, and eluted pellet. 400ul sample of the entire spiked homogenate was sampled. Reference samples were then centrifuged for 2500g for 10 minutes at 4°C and supernatant was collected for culture analysis and quantification of viable virus and used as reference points for samples treated with disinfectant. For reference enveloped virus samples, 2ml of 3% beef extract, 0.05M glycine was added to the pellet and vortexed for 3 minutes. For reference non-enveloped virus samples, 1 ml PBS and 1ml of Vertrel XF (Dupont) was added to the pellet and vortexed for 3 minutes for an organic solvent extraction. All samples were centrifuged for 2500g for 10 minutes at 4°C and eluted pellet buffer (supernatant) was collected. Spiked homogenate, supernatant, and eluted pellet samples were quantified for viable MS2 and phi6 phage. Remaining volumes of reference enveloped, and non-enveloped samples were extracted for viral RNA/DNA using the Qiagen MinElute viral RNA/DNA spin kit according to the manufacturer's instructions. qPCR and RT-qPCR was run on HAdV-2 and OC43 spiked homogenate,

supernatant, and eluted pellet to determine viral genomic partitioning in the untreated reference samples prior to disinfection. qPCR was run alongside a serially diluted stock standard curve to relative genomic copies compared with spiked titer samples of the spiked homogenate, supernatant, and eluted pellet.

After adjusting for differences in volume between the three sample portions viral culture (phi6, MS2 and HadV2) and qPCR relative genome copies for HAdV-2 and HCoV-OC43 were calculated to determine total genome copies adjusting for volume. Since time spent in suspension was not accounted for, each experiment was analyzed and described separately due to differences in batches and experiment days. For quality control purposes, all samples were run in duplicates and plates included molecular grade water no template controls, unseeded background wastewater RNA/DNA, spiked titers, and a calibration curve using serially diluted HAdV-2 stock or HCoV-OC43 constructed RNA (Vijgen, Keyaerts et al.). For OC43 RT-qPCR runs (n=3) averaged efficiencies of 100% ±1% with $R^2 = 0.99$ using a constructed RNA standard calibration curve. For HAdV-2 qPCR runs (n=3) averaged efficiencies of 96% ±2% with $R^2 = 0.99$.

Results & Discussion

Table 4.3 MS2 disinfection using QAD for 5, 10, and 20 minutes of contact time using concentrations of 0, 0.4%, 1%, and 1.5% QAD.

Contact time	Treatment	Mean Log_{10} ± 95% CI	σ_x	N	LRV± 95% CI	>4 LRV
5 min	Reference	8.6 ±0.2	0.18	5	--	
	0.4%	8.3 ±0.04	0.05	5	**0.31 ±0.2**	
	1%	8.3 ±0.07	0.08	5	**0.31 ±0.2**	
	1.5%	8.1 ±0.2	0.18	5	**0.54 ± 0.2**	
10 min	Reference	8.2 ±0.06	0.07	5	--	
	0.4%	8.0 ±0.1	0.12	5	**0.20 ± 0.1**	
	1%	8.3 ±0.03	0.04	5	**0.04 ±0.05**	
	1.5%	7.8 ±0.2	0.22	5	**0.5 ±0.2**	
20 min	Reference	8.3 ±0.04	0.05	5	--	
	0.4%	8.3 ±0.06	0.07	5	**0.05 ±0.07**	
	1%	8.1 ±0.06	0.07	4	**0.18 ±0.07**	
	1.5%	7.9 ±0.05	0.05	5	**0.38 ±0.06**	

Disinfection trials on a suite of enveloped, non-enveloped, and RNA/DNA genomic viruses using a quaternary ammonium disinfectant (QAD) and a pH raising potassium hydroxide detergent (KOH-D) demonstrated a sharp divide between disinfection between non-enveloped viruses and enveloped viruses in a research animal facility (Figure 4.1). We found that enveloped, RNA viruses phi6 and SRV-2 were readily inactivated by >4 log_{10} reductions using the QAD and KOH-D, respectively. MS2 and HAdV-2 were relatively resistant to both disinfectants tested where KOH-D appeared to provide some reductions at highest tested concentrations 2% and 4% KOH-D. KOH-D disinfection testing was discontinued after initial HAdV-2, MS2, and SRV-2 testing in this research because treated wastewater was above the allowed pH (>10.0) for local sewage discharge and unfeasible to neutralize (data not provided). Due to the difficulty of using and neutralizing KOH-D, WaNPRC quickly pivoted away from using KOH-D in their facilities and ended further experimentation. The varied efficacy of both KOH-D and QAD between viral pathogens and surrogates in animal facility wastewater, suggests a need for individualized testing on viral vectors or corresponding surrogates to better understand appropriate disinfection protocols required for containment of recombinant viruses and viral vectors.

MS2 QAD disinfection resulted in little to negligible decreases in viable virus despite high levels of seeded virus (~10^8) where MS2 experienced as 0.5, 0.5, and 0.4 Log_{10} reductions at 5-, 10-, and 20-minutes of contact time with 1.5% QAD concentrations (Table 4.3). QAD disinfection of HAdV-2 disinfection data is forthcoming but expected to have low or negligible disinfections levels due to similarities in structure with MS2 and similar disinfection trends seen in KOH-D disinfection profiles (Table 4.2). QAD MS2 disinfection profiles across contact time and concentration were the same (Figure 4.1 & Table 4.3) where any measured disinfection levels likely occurred within 5 minutes of contact time. Previous research on surface disinfection of viral pathogens, including MS2, have found similar disinfection results where quaternary ammonium disinfectants provided only modest reductions (~3.3 log_{10} reductions) at 10x recommended working concentration of the disinfectant (Solomon, Fino et al. 2009) and at significantly higher concentrations than tested in this research. These results are not

surprising considering that quaternary ammonium disinfectants inactivate pathogens via lipid membrane dispersion, making non-enveloped viruses resistant to detergents and lipophilic dispersants such as quaternary ammonium compounds.

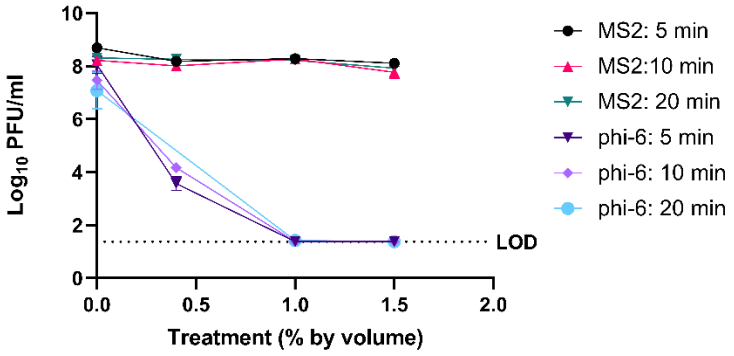

Figure 4.1 Quaternary ammonium disinfection of Phi-6 and MS2 bacteriophages for 5-, 10-, 20- minute contact times and 0.4, 1, and 1.5% disinfectant volumes.

Table 4.4 Phi6 disinfection using QAD for 5, 10, and 20 minutes of contact time using concentrations of 0, 0.4%, 1%, and 1.5% QAD.

Contact time	Treatment	Mean Log_{10} ± 95% CI	σ_x	N	LRV± 95% CI	>4 LRV
5 min	Reference	8.0 ±0.3	0.3	5	--	
	0.4%	3.6 ±0.2	0.3	5	**4.5 ±0.4**	Yes
	1%	< 1.4 ±0.0	0.0	5	**>6.7 ±0.3**	Yes
	1.5%	< 1.4 ±0.0	0.0	5	**>6.7 ±0.3**	Yes
10 min	Reference	7.5 ±0.30	0.34	5	--	
	0.4%	4.2 ±0.1	0.11	5	**3.30 ±0.32**	
	1%	< 1.4 ±0.0	0.00	5	**> 6.1 ±0.3**	Yes
	1.5%	< 1.4 ±0.0	0.00	5	**> 6.1 ±0.3**	Yes
20 min	Reference	7.1 ±0.6	0.68		--	
	0.4%	*Data unavailable*				
	1%	1.4 ±0.1	0.10		**5.7 ±0.1**	Yes
	1.5%	< 1.4 ±0.0	0.00		**> 5.7 ±0.1**	Yes

Phi6 bacteriophage was readily inactivated by QAD where the lowest tested concentration 0.4% resulted in 4.48, 3.30, and 5.66 log10 reductions across 5, 10, and 20 minutes of contact time (Table 4.4). Identical disinfection profiles (Figure 4.1) indicates that inactivation occurs under 5-minutes of contact time. This could be due to added vortexing step in disinfection trial and rapid distribution of the QAD in

the wastewater but might not be scalable to larger catchment troughs in the animal facilities requiring the maximum tested contact time. Cytotoxicity in cell culture for HCoV-OC43 meant that methodological ability to observe QAD coronavirus disinfection capability in animal wastewater was not possible. While phi-6 phage is not considered to be an appropriate surrogate for coronaviruses in the environment other enveloped viruses such as Ebola (Whitworth, Mu et al. 2020) are more accurately reflected in these disinfection trials.

Expectedly, SRV-2 had the largest reductions with KOH-D disinfection, average 2.7 and 4.6 $\log_{10}$ reductions using 0.7% and 1% final KOH-D concentration with 10 minutes of contact time (Table 4.7). This was the only virus to have reductions > 4 $\log_{10}$ at any level of KOH-D in viable virus and nucleic acids (RT-qPCR) significant enough to merit further investigation of nucleic acid degradation. Reductions due to disinfectants were determined using RT-qPCR with confirmation of virus viability through titration on to Raji cells (Table 4.7 & Figure 4.2). Enumeration of viable virus on Raji cells indicated that SRV-2 was inactivated to non-detectable levels at only 1% KOH-D, but significant decreases in viability occurred at 0.7% KOH-D as well (Figure 4.2). This level of disinfection for SRV-2 at 0.7% and 1% concentrations of KOH-D, demonstrates that both viable and nucleic acids disinfection process of animal waste containing SRV-2 were eliminated in this study. Additional validation work regarding recombinant nucleic acid containment would have to look at specific recombinant genes and qPCR sensitivity in these settings.

Table 4.5 Human Adenovirus-2 Disinfection using 0.7, 1, 2, and 4% KOH-D for 10 minutes contact time

Treatment	Reference Mean Log_{10} ± 95% CI	Mean Log_{10} ± 95% CI	σ_x	N	LRV± 95% CI	Mean LRV	>4 LRV
0.7%	5.4 ±0.1	5.4 ±0.1	0.11	3	0.07 ±0.2	**0.07**	
1%	5.4 ±0.1	5.4 ±0.4	0.31	3	0.07 ±0.4	**0.07**	
2%	5.6 ±0.4	3.8 ±0.4	0.31	3	1.7 ±0.5	**1.3**	
	5.4 ±0.1	4.5 ±0.2	0.20	3	0.9 ±0.3		
4%	5.6 ±0.4	3.6 ±0.3	0.23	3	2.0 ±0.4	**2.2**	
	5.4 ±0.1	3.1 ±0.4	0.35	3	2.3 ±0.4		

Adenovirus and MS2 bacteriophage experienced less die-off during disinfectant trials than SRV-2 (Table 4.8) indicating that additional contact time, temperature or secondary treatment is required for proper disposal and release into sanitary sewers. MS2 had average $\log_{10}$ reductions of 0.48, 0.56, and 2.19 at disinfectant concentrations tested with KOH-D concentrations of 0.7%, 1%, and 2% respectively (Table 4.8). MS2 has similar characteristics to many non-enveloped RNA viruses and was specifically chosen for this study because of its broad application in disinfection and wastewater treatment literature as an enteric virus surrogate (Kott, Roze et al. 1974). HAdV-2, experienced average $\log_{10}$ decreases of 0.07, 0.07, 1.3, and 2.2 TCID50 mL^{-1} following 0.7%, 1%, 2% and 4% KOH-D treatments for 10 minutes of contact time at room temperature (Table 4.5). HAdV-2 experienced the least amount of die-off due to disinfection of all viruses tested, the most substantial reductions in HAdV-2 occurred at 2% and 4% KOH-D concentrations where geometric average $\log_{10}$ reductions were 1.3 and 2.2 TCID50 mL^{-1}, respectively. HAdV-2 is a non-enveloped enteric virus with broad interest in disinfection and wastewater treatment but has a ~15kb DNA genome (Gingeras, Sciaky et al. 1982) Similar to MS2, in this another research adenoviruses appear to be resistant to QAD disinfection (Rutala William, Peacock Jeffrey et al. 2006) and partly-resistant to KOH based disinfectants (Table 4.5). For both MS2 and HAdV-2, viability was not substantially reduced, and qPCR was not performed to determine nucleic acid elimination from wastewater samples. Concentrations of KOH-D that were successful in achieving measurable reductions (Table 4.6 & Table 4.7) in MS2 and HAdV-2 are on the higher end of the manufacturer's suggested usage of this disinfectant of 0.4-4.7% at varied pH, temperatures, and soil loads.

Table 4.6 MS2 Disinfection using KOH-D for 10 minutes contact time

Treatment	**Mean $\mathbf{Log_{10}}$ ± 95% CI**	**σ_x**	**N**	**LRV± 95% CI**	**>4 LRV**
Reference	8.9 ±0.2	0.15	4	--	
0.7%	8.4 ±0.1	0.12	5	**0.5 ±0.2**	
1%	8.4 ±0.1	0.10	5	**0.5 ±0.2**	
2%	6.7 ±0.3	0.29	5	**2.2 ±0.3**	

The NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules has strict guidelines for permissions and biosafety levels for recombinant viruses and nucleic acids but fail, to provide treatment levels or testing requirements for waste. Our study found varying efficacy of KOH-D and QAD on a suite of viral pathogens and surrogates as a potential pre-treatment step prior to release into a sanitary sewer. Our research has arbitrarily set a 4-$\log_{10}$ reduction value to differentiate between high disinfections levels seen in SRV-2 and phi6 and low disinfection seen in MS2 and HAdV-2 disinfection levels, which is also in line with EPA product performance test guidelines for disinfectants in water (US EPA: OCSPP 810.2600, 2012). We believe that experimental differences between viruses were likely due to the viral structure (Table 4.1), where enveloped viruses were readily inactivated by KOH-D and QAD. High alkaline conditions observed KOH-D in this study likely denatured the membrane envelope and proteins necessary for attachment, cell entry or replication for SRV-2 and eliminated nucleic acids. Whereas QAD disinfection worked to dissolve lipid membranes of phi6 and was not consumed by wastewater substrate because of its susceptibility to oxidant demand. Minimal HAdV-2 reductions were likely due to its non-enveloped capsid and large DNA genome, which has been demonstrated to be resistant to other forms of disinfection such as UV and would require much more stringent disinfection routines. Reference controls helped to accommodate for substrate heterogeneity and composition; however, the heterogeneous nature of the combined fecal wastewater, organic material, humic acids, and other non-target compounds likely hindered more dramatic disinfection in non-enveloped viral targets in KOH-D disinfection.

Table 4.7 SRV-2 Disinfection using KOH-D for 10 minutes contact time

Treatments (n)	C_q	Mean C_q	Log_{10} Reduction
Ref: Media (2)	20.50 23.38	21.94	--
0.7% (2)	31.80 28.29	30.05	**-2.7**
1% (2)	NA* 35.64	35.64	**-4.6**

*No amplification

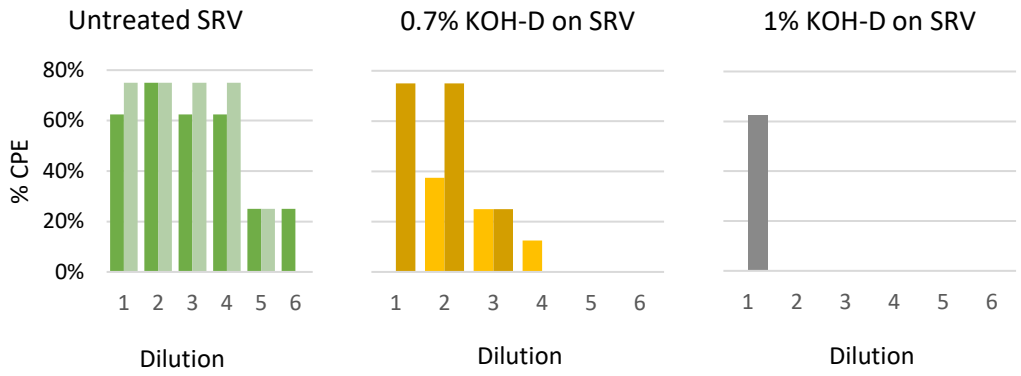

Figure 4.2 Titration of 10-fold dilutions of untreated and 0.7% and 1% KOH-D treated SRV-2 onto Raji cells. Cytopathic effects (CPE) were observed across cell wells due to viral replication and viability.

Table 4.8 Log_{10} change across HAdV-2, MS2, and SRV-2 KOH-D disinfection

Treatment	MS2	HAdV-2	SRV
0.7%	-0.5	-0.07	-2.7ǂ
1%	-0.5	-0.07	-4.6ǂ
2%	-2.2	-1.3	--*
4%	--*	-2.2	--*

* Experiment not conducted
ǂ Calculations based on RT-qPCR data

To better understand partitioning of viruses within our animal wastewater microcosms, we conducted a thorough investigation of seeded viruses in spike homogenate, supernatant, and eluted pellet buffer of experimental reference samples. Volume based measurements were consistent between three different experiments resulting in 75% supernatant or liquid portions with 23-25% of total volumes representing the solids and pellets (Table 4.9). This was consistent across our experiments including a batch of wastewater requiring dilution to facilitate pipetting during experimentation. For MS2 and phi6 culture-based analysis, phage readily partitioned into supernatant of our microcosms (Figure 4.3 A & C), where volume adjusted PFUs found in the supernatant and pellet never exceeded the seeded amount. This is consistent with two other partitioning studies looking at wastewater (Ye, Ellenberg et al. 2016), synthetic sludge, and anaerobically digested sludge (Titcombe Lee, Pruden et al. 2016). HAdV-2 viral DNA analyzed using qPCR (Figure 4.3D) yielded similar results where viral DNA primarily partitioned to the liquid portions of the microcosms and 0-15% of viral DNA partitioning to the solid portions of the

samples. Viral RNA partitioning of HCoV-OC43 (Figure 4.3B) resulted in viral RNA 2-3 orders of magnitude higher than seeded spike homogenate values across both supernatant and eluted pellet, where eluted pellets accounted for >98% of total viral RNA in a single sample. While OC43 results were highly unusual and not consistent with phi6 culture results, which were seeded into the same microcosms, a similar result was reported in (Titcombe Lee, Pruden et al. 2016) where supernatants and pellets had 2-3 orders of magnitude more than seeded phi6 phage values where increases were attributed to phage replication on native bacterium in biosolids. Since our experiments used pasteurized primate wastewater and this observation only occurred in HCoV-OC43 using qPCR which requires mammalian host cells for replication, we believe this is due to the high ratio of free genomic RNA to viable virus in our seeded viral stocks, where nucleic acids could partition to solid substrates in the samples compared to encapsulated virions. Additional work needs to be done to determine the concordance between qPCR and culture results in these systems to better interpret our unusual results.

Other reasons why this same effect was not seen in HAdV-2 qPCR could be due to the pellet elution and processing, where an organic solvent extraction was performed on non-enveloped viruses using Vertrel XF but resuspension of viruses in beef extract was performed on enveloped viral samples. Or due to inefficient nucleic acid extraction of spike homogenate resulting in a lower seeded. These results require RT-qPCR follow up on phi6 samples to determine concordance with viable virus and viral RNA partitioning in samples. While unresolved these results have implications seen in wastewater surveillance of SARS-CoV-2 where disproportionally higher molecular signals are seen in settled or centrifuged solids (Graham, Loeb et al. 2021). One major limitation of these partitioning experiments was that time post seeding was not a consideration and data for each individual experiments varied significantly and therefore were grouped together.

Table 4.9 Distribution of solids and liquids in primate wastewater experiments

	Enveloped Viral Microcosms (n=15)			**Non-Enveloped Viral Microcosms (n=12)**	
	Supernatant	Pellet		Supernatant	Pellet
Avg Vol ± 95% CI (mL)	1.50 ± 0.04	0.50 ± 0.04	Avg Vol ± 95% CI (mL)	1.46 ± 0.07	0.54 ± 0.07
% Vol	75.1%	24.9%	% Vol	73%	27%

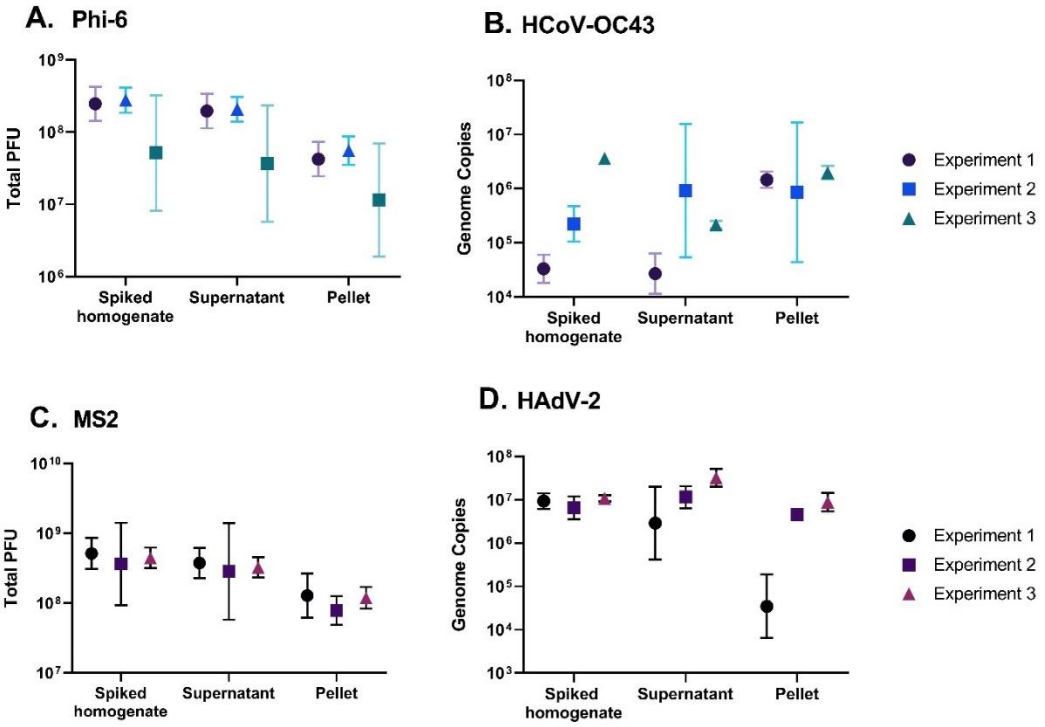

Figure 4.3 **Viral partitioning data.** Three representative experiments with volume adjusted plaque forming units or genome copies in spiked homogenate, supernatant, and eluted pellet liquid for A) Phi-6 plaque forming units, B) genome copies of HCoV-OC43, C) MS2 plaque forming units, and D) HAdV-2 genome copies.

This study compared two disinfectants against two pathogens and two viral surrogates under standardized conditions highlighting the need to evaluate disinfection of experimental viral pathogens and replicant competent viral vectors in operational animal research facility wastewaters. Additionally, determining acceptable reduction levels required for recombinant nucleic acid or viral vector disposal is not quantified in NIH recombinant nucleic acid guidelines and is likely dependent on viral type, levels of demonstrated viral shedding from test animals, research purpose, local regulations, institutional biosafety

committee requirements, and additional risk associated with the virus or genes of concern. Other studies using viral vectors or recombinant nucleic acids and the biosafety of viral vectors have found that heating, and detergents are usually sufficient to inactivate lentiviral vectors and adeno-associated vectors (Porter and Lyons 2002, Collins, Reuter et al. 2017, Kriesel, Stegmann et al. 2020) but testing for recombinant gene survival in the environment via molecular methods are not well established and are highly dependent for each independent line of investigation. Using HAdV-2 and MS2 as surrogates for experimental pathogens and recombinant viral vectors is a conservative approach for evaluating the most environmentally resistant viral vectors. SRV-2, phi6, and HCoV-OC43 experimentation highlights the range of responses to different disinfection scenarios that occur in animal research.

In conclusion, the use of QAD or KOH-D as an off-label pre-treatment step prior to release into a sanitary sewer could be a feasible method for inactivating enveloped viruses, but more work needs to be done by to define treatment standards and containment policies. Our experiments show reduced infectivity of enveloped viruses during disinfection trials in primate facility wastewater with heavy organic loads on experimentally infected viruses on the most labile viruses with envelope membranes, such as SRV-2, phi6 or HCoV-OC43. KOH-D disinfection was effective against SRV-2 but created a pH hazard that proved difficult to overcome during routine operations of the primate center and was not implemented for long term use. Partitioning experiments of seeded viruses validate use of sample supernatant to look at viral viability during disinfection trials. Additional research needs to be done to better understand the discrepancies found in HCoV-OC43 RT-qPCR data. Differences in viral reductions between phi6, SRV-2, MS2 and HAdV-2 highlight the differences in disinfection susceptibility of different viruses during disinfection and cleaning routines. This could help guide future decision making either by adopting a conservative marker for protocol testing (MS2 or HAdV-2) or by evaluating each virus of interest in these settings to prevent occupational risks to animal care researchers and unintended release into environment. Next steps include investigating disinfection mechanisms of SRV-2 and disinfection conditions necessary for recombinant protein inactivation. Additionally, conditions or treatments necessary to inactivate MS2 and HAdV-2 in this substrate need to be worked on and verified.

Methods such as MALDI-TOF MS and SDS-PAGE can be used to analyze alterations in viral attachment, replication, viral proteins, and genome replication would provide a more complete picture regarding effective strategies for neutralizing other pathogens and recombinant genes that are in use in animal research facilities.

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5. Evaluation of Theiler's Murine Encephalitis Virus in Intestinal Enteroid Cultures

Introduction

Theiler's Murine Encephalitis Virus (TMEV) is a murine cardiovirus used to create acute seizures, epileptic disorders, and multiple sclerosis in murine models for biomedical research and to screen for pharmacological agents in these fields (DePaula-Silva, Hanak et al. 2017). Like other Picornaviruses, TMEV is shed in the feces, infects the gastrointestinal tract, and can disseminate to neural tissue occasionally causing an array of neurological comorbidities (Bröer, Käufer et al. 2016). Few research studies have been conducted to understand the role of the GI tract in TMEV infection and neurological disease progression. There is growing research indicating that dissemination of enteric pathogens to the central nervous system is highly dependent on infection mechanisms in the GI tract with ties to microbiome interactions, host responses, and the viral lifecycle (Luethy, Erickson et al. 2016).

Of the studies conducted on TMEV infection in the GI tract, two cell targets have been identified as important for TMEV infections. Goblet cells were identified through an *in vivo* study looking at a closely related Theilovirus in rats (Drake, Besch-Williford et al. 2011) and enterocytes were identified in an *ex vivo* viral overlay and attachment experiment (Tsunoda, Libbey et al. 2009) indicating intestinal epithelium can be infected in mice and the initiation site for viral infection and disease progression. An additional study has been conducted on TMEV intracerebral infections and the murine microbiome where findings indicate that active neurological viral infections are associated with significant shifts in intestinal flora (Carrillo-Salinas, Mestre et al. 2017). These studies highlight the importance of the GI tract and development of neurological disease in TMEV and aligns with research of neurovirulent human pathogens such as poliovirus and enterovirus-71 (Kuss, Etheredge et al. 2008). Understanding mechanisms behind viral infection of the GI tract is paramount to understanding disease pathogenesis and developing therapeutics and vaccines.

Utilization enteroid cell lines derived from intestinal stem cells elicited several important host-pathogen breakthroughs and are a valuable research model for *in vitro* host- pathogen studies across viral, bacterial, and parasitic agents. Enteroid cells lines have been developed from multiple different species including humans and mice where harvested intestinal stem cells can be proliferated and differentiated into a number of different cell types that are represented in the GI epithelium. When suspended in collagen rich environment, intestinal enteroids can also reproduce important physiological features such as crypts and villi. This reductionist model of the GI tract is advantageous in that they can be maintained for many months without significant alterations to cell types, maintain cell polarization, and can perform specific cellular functions that are not represented in immortalized cell lines. Infectious disease research using enteroids have allowed for better understanding of a number of different viral pathogens including norovirus, echovirus, enterovirus-71 and adenovirus.

Given the interest in mechanisms of TMEV GI infection and availability of murine intestinal enteroids, the objective of this research was to evaluate the utility of murine intestinal enteroids as model for TMEV infection in the GI tract and highlight the need for further research on TMEV infection in the GI tract. To do this we evaluated viral replication via RT-qPCR of four different TMEV strains (BeAn, DA, GDVII, and WW) alongside Mouse Adenovirus-2 exposed to murine enteroid cultures. Further experiments with repeated viral passaging and using JAK/STAT inhibitor, ruxolitinib, were done to encourage viral replication. Enteroid cultures that were exposed and unexposed to virus were characterized for key intestinal gene markers via gene expression assays.

Methods

Viruses and host cells. TMEV strains DA, BeAn, GDVII, and WW were all generous gifts from Dr. Robert Fujinami at the University of Utah. All strains of TMEV were grown on confluent monolayer BHK-21 cells (ATCC CCL-10) with DMEM, 2% FBS, and Gen/Kan for 48 hours until distinctive CPE was observed. Infected flasks were frozen and thawed 3x, prior to organic solvent extraction using Vertrel XF. Virus stocks were stored at -80 °C and quantified by plaque assay on BHK-21 cells.

Quantification of virus stocks and samples were done via plaque assay using confluent BHK-21 cells in 6-well plates (Greiner). Wells were overlaid with 1.6% Avicel, DMEM, 1% Gen/Kan, 3.5% Sodium Bicarbonate, 10mM HEPES, Nystatin, and 2% FBS. After 4 days post infection, monolayers were fixed and stained with 20% Methanol and crystal violet. Visible plaques were counted and quantified.

Mouse Adenovirus-2 (MAdV-2) and CMT-93 cells were gifts from Dr. Jason Smith from the University of Washington. MAdV-2 stocks were grown on confluent layers of CMT-93 cells in DMEM, 2% FBS, and 1% Gen/Kan for 48 hours until visible CPE. Infected flasks were frozen and thawed three times. Lysate then underwent an organic solvent extraction using Vertrel XF (Dupont), where equal volumes of Vertrel XF were added to lysates, vortexed for 3 minutes, and centrifuged at 2500g for 15 minutes at 4 °C. Supernatant was collected and stored at -80 °C until use in experiments. MAdV-2 stocks were enumerated using an endpoint dilution method on CMT-93 cells for 72 hours to determine TCID50/ml.

Murine Intestinal enteroid lines were harvested as incidental tissues from the labs of Drs. Melissa Barker-Haliski (University of Washington, Dept. of Pharmacology) and Terrance Kavanagh (University of Washington, Dept. Environmental and Occupational Health Sciences) according to previously published methods by Sato et al and in accordance with University of Washington IACUC procedures for respective labs (Sugimoto and Sato 2017). Enteroid lines from 10 cm of distal small intestine CF-1 male mice and cultured using IntestiCult Intestinal Organoid Growth Medium (Stem Cell Technologies, Vancouver, Canada) and suspended in Matrigel with media changes and passaging every 3-4 days.

Enteroid-Viral Growth Experiments. Enteroid intestinal cells that were 3-4 days after passaging were broken up using a 25-gauge syringe and isolated by slow speed centrifugation. Isolated intestinal cells were then exposed to 10^7 PFU of TMEV for 1 hour for attachment at 4°C while in IntestiCult organoid growth media + J1 (0.5mM). After 1 hour of attachment, cells were isolated via centrifugation, supernatant was removed, and cells were washed with PBS. This was repeated twice to remove

unattached virus. MAdV-2 infections from the same set enteroids were also performed along side TMEV infections as a positive control to ensure that infection protocols were performed correctly.

Virus exposed enteroid cells were then resuspended in Matrigel and growth media and 50 ul Matrigel, media and cell drops were plated in a 24-well plate. Wells with Matrigel were allowed to solidify for 15 minutes at 37C prior to the addition of 0.5ml of growth media. Wells were harvested at times 0-, 24-, 48-, 72-, and 96- hours post viral exposure and stored at -80C until RNA/DNA extraction. Each TMEV enteroid exposure was replicated 2-3 times for each of the four strains along with MAdV-2 to ensure viral-cell exposure could result in a successful infection.

Enteroid/TMEV Passaging. To extend growth period for TMEV in enteroids, CF-1 murine enteroids at 3-4 days post passaging were exposed to TMEV-DA and plated as described above. Cell lines exposed to TMEV were passaged every three days for four subsequent passages (12 days total). Whole well samples (overlay media and cells) were taken at each initial plating. After the 4-day growth period separate overlay media, cell, and supernatant samples were collected go determine viral increases over time. RT-qPCR was run on initial samples, supernatant, and collected cells and compared to baseline to determine if an extended growth period resulted in detectable viral replication.

JAK/STAT Inhibitor treated enteroid viral experiment. Previous experiments have demonstrated increased viral replication in the presence of a JAK/STAT inhibitor ruxolitinib in enteroid cultures to reduce endogenous interferon response (Good, Wells et al. 2019, Kolawole, Mirabelli et al. 2019, Hosmillo, Chaudhry et al. 2020). To coax TMEV growth in murine enteroids, CF-1 murine enteroids were pre-treated with 10µm ruxolitinib (Cayman Chemical) in growth media for 3 hours prior to exposure of TMEV-DA and GDVII strains as described above. Viral RNA from timepoints 0, 24, 48, 72, and 96-hours post exposure was quantified using RT-qPCR and compared to baseline to determine change over time.

qPCR and gene expression. Frozen viral samples were extracted for viral RNA/DNA using Qiagen Minelute viral RNA/DNA spin kit according to the manufacturer's instructions. Viral RNA and DNA was quantified using RT-qPCR and qPCR from previously described primers and probes (Yuan, Wang et al. 2016). For RT-qPCR of viral RNA, BioRad Universal Probes One-step mix (BioRad) was used with final concentrations of both the forward and reverse primers at 400nM and Fam-BQ1 probe at 200nm with the following cycling conditions: 50 °C cDNA step for 10 minutes, 95 °C denaturing and 60 °C elongation steps for 40 cycles on a BioRad CXF96 machine. MAdV-2 DNA samples were quantified using previously described qPCR assay (Wilson, Bromme et al. 2017) using SsoAdvanced SYBR BioRad Mix. Samples were quantified relative to zero time points using a standard curve to determine fold difference over time.

Samples that were processed and analyzed for gene expression markers included CF-1 intestinal enteroids used in experiments, including viral exposed and untreated samples. To ensure ruxolitinib decreased cellular interferon activity, ruxolitinib treated and non-treated enteroids were analyzed for changes in gene expression for at 3 and 48 hours post ruxolitinib treatment.

For all gene expression assays, total RNA was for samples using Trizol LS followed by the Purelink RNA kit (Invitrogen). One microgram of total RNA was reverse transcribed in a 20ul reaction using the SuperScript VILO cDNA synthesis kit according to the manufacturer's instructions and diluted to 100ul prior to downstream qPCR analysis. Sso Advanced SYBR Mix or iTaq Universal SYBR green mix (BioRad) was used to quantify gene expression across gene markers and housekeeping genes (Table).

Table 5.1 Gene expression assay for murine intestinal cell markers

Assay	Gene Target	Forward Primer	Reverse Primer	Reference
ChgA	Ms Chromogranin A- Enteroendocrine	TTCCATGCAGGCTACAAAG	GTCTTTCCATCTCCATCCAC	(Petersen, Reimann et al. 2014)
Lyz1	Lysozyme 1- paneth cells	GGAATCGGATGGCTACCGTGG	CATGCCACCCATGCTCGAAT	(Petersen, Reimann et al. 2014)
Lgr5	Luecine-rich repeat containing G protein-couples receptor 5	CTTTGACACACATTCCCAAG	AAATTCTGTAGCGCTTCTC	(Petersen, Reimann et al. 2014)
I-Fabp	Ms intestinal fatty acid binding protein- Enterocyte markers	CGGCACGTGGAAAGTAGACC	AATGGTCCAGGCCCCAGTGA	(Petersen, Reimann et al. 2014)
Itf	Ms intestinal trefoil factor- Goblet cells	CCTCTGGCTAATGCTGTTG	CAGTCCACTCTGACATTTGC	(Petersen, Reimann et al. 2014)
RPL5	Ms housekeeping	GGAAGCACATCATGGGTCAGA	TACGATCTTCATCTTCCTCCATT	(Wilson, Tocchi et al. 2015)
B2m	Ms housekeeping	CTGGTGCTTGTCTCACTGAC	GTTCAGTATGTTCGGCTCC	(Petersen, Reimann et al. 2014)

Results

TMEV exposure to CF-1 enteroids for 96-hours with 24-hour sampling increments led to steady decreasing levels of viral RNA compared to inoculated virus for DA, GDVII, WW, and BeAn strains of TMEV (Figure 5.1). In these same experiments MAdV-2 viral DNA consistently increased almost 100-fold over baseline and leveling off at 48-hours (Figure 5.1). Negative control enteroids wells had no detectable viral RNA/DNA. Enteroids infected with MAdV-2 were included using the same preparations of murine enteroid for each TMEV experiment.

Further exploration of different methods to improve conditions for TMEV replication in enteroids involved extending the growth period for TMEV in enteroids and introducing a JAK/STAT inhibitor ruxolitinib. Passaging TMEV-DA with murine enteroids allowed for TMEV-DA to be exposed to a continuous enteroid culture for 12 days. TMEV-DA viral RNA decreased steadily below the expected decreases for sub-passaging dilution for 3 passages. At the fourth passage (days 9-12), TMEV viral RNA

appears to level off and increase to levels above the third passage. Experiment had three replicates for each passaging point and was replicated only once.

Introduction of an interferon and JAK/STAT inhibitor, ruxolitinib, using TMEV strains GDVII and DA over 96-hour exposure with 24-hour sampling time points showed that GDVII viral RNA steadily decreased over 96-hours. TMEV-DA viral RNA increased over 48-hours by as much an 8-fold compared to baseline viral RNA but decreased to baseline and below baseline at 72- and 96-hours post infection (Figure 5.1).

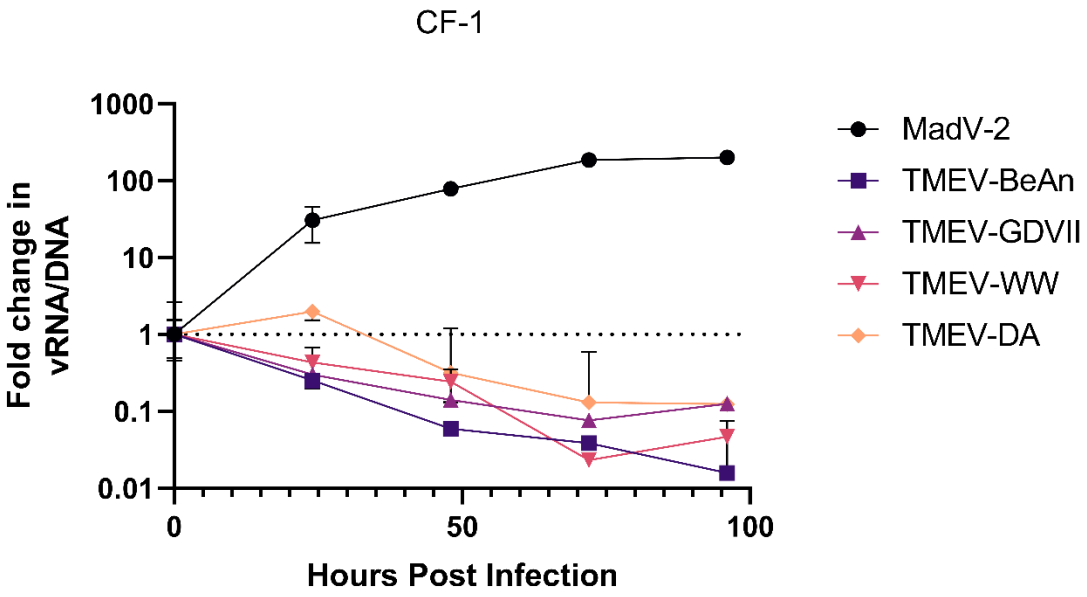

Figure 5.1 Change in viral RNA/DNA for MAdV-2 (black) or TMEV exposed to CF-1 murine intestinal enteroids.

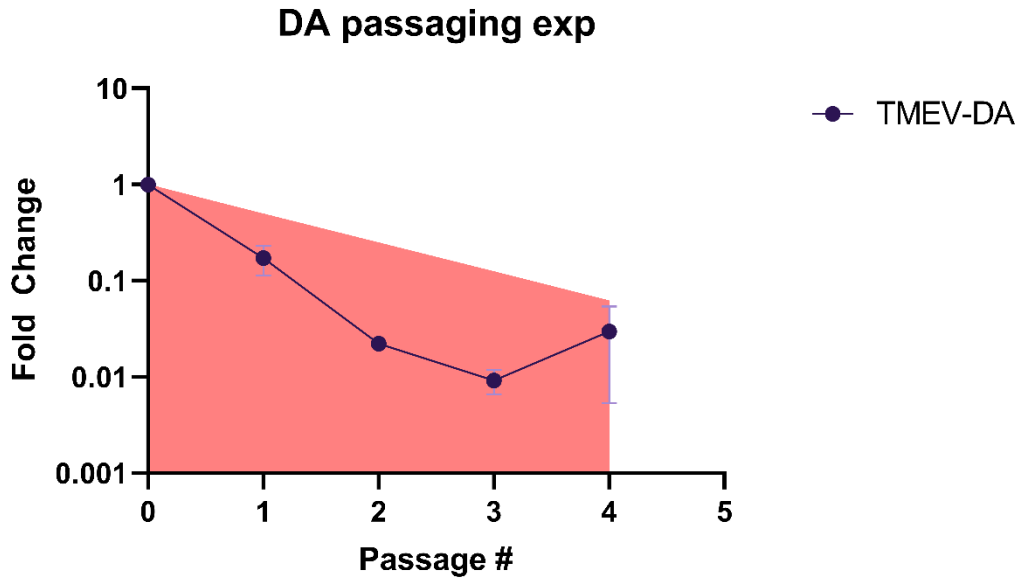

Figure 5.2 Serial passaging of TMEV-DA in murine enteroids continuously for 12 days with sub-culture passaging every 3 days. Red shading indicates expected dilutional decreases due to sub-passaging.

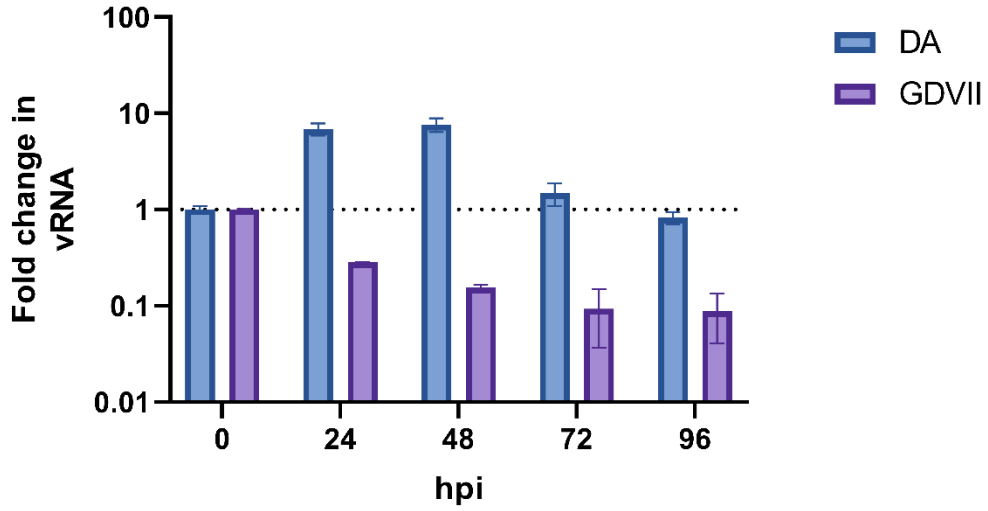

Figure 5.3 Change in viral RNA from TMEV strains GDVII (purple) and DA (blue) in CF-1 enteroids pretreated with ruxolitinib over 96 hours post infection (hpi).

Discussion

Additional research is needed to determine if TMEV can replicate in wild-type murine intestinal enteroids (Figure 5.1). Previous work on TMEV *ex vivo* and *in vivo* has suggested that enterocytes or goblet cells are the primary targets for this virus (Tsunoda, Libbey et al. 2009, Drake, Besch-Williford et al. 2011) but this body of research suggests a more host-viral interaction despite the presence of overlapping cell types that readily infect MAdV-2 (Wilson, Bromme et al. 2017). Successful replication of Murine Adenovirus-2 (Figure 5.1) in side-by-side enteroids experiments with TMEV demonstrated user competency of viral-enteroid methods, indicating that TMEV infection in intestinal enteroids presents a more complicated viral-host dynamic requiring further research.

Previous work using enteroviruses with human intestinal enteroids have shown that interferon types I and III restrict enterovirus replication (Good, Wells et al. 2019, Hosmillo, Chaudhry et al. 2020), indicating possible restriction of TMEV due to tissue level immune responses in this murine model. This is in contrast to other enteric viruses such as rotavirus that have mechanisms to manipulate interferon response (Saxena, Simon et al. 2017). This research showed that the incorporation of JAK/STAT inhibitor into our experiments used to reduce immune response of tissues and increase TMEV replication in enteroid cultures had differing responses with GDVII and DA strains (Figure 5.3). With data forthcoming on the use of ruxolitinib on gene expression during serial passaging experiments to determine if it effective at reducing interferon response in these experiments to better understand differences in viral RNA between DA and GDVII strains over the 96-hour exposure time (Figure 5.3). Viral RNA of TMEV-GDVII consistently declined over 96-hour exposure, while TMEV-DA saw 6 & 8-fold increases for the first 48 hours and then declined to baseline at 72 and 96-hours post exposure to enteroid cultures. These results suggest a couple of different scenarios: 1) spurious increases due to cell or viral preparation with no viral replication, 2) abortive viral RNA replication occurred but was inhibited when after drug was consumed/degraded *in vitro*. In either scenario further experiments need to be done to replicate results, probe for viral RNA replication or viable virions, and to determine the role of

ruxolitinib in these increases. Studies looking at host response to Theiler's murine virus infection has found strain level differences between neurovirulent strains which result in fast non-persistent viral infections, and non-neurovirulent strains, resulting in persistent viral infections. These differ significantly between mice strains and have been attributed to their interferon responses (Bröer, Käufer et al. 2016). RNA viruses, such as TMEV, are notoriously sensitive to type I and III interferon responses where viral replication is restricted (Lancaster and Pfeiffer 2010, Good, Wells et al. 2019), and tissue dissemination limited but it is unclear if there are other reasons for the lack of replication seen in this work.

Extended growth periods for TMEV-DA during serial passaging experiment (Figure 5.2), showed steady decreases in viral RNA over the first three sub-culturing points (0-9 days) suggesting viral decay without growth or viral adaptation. There was a leveling off and slight increasing in viral RNA at the fourth passage (9-12 days) which could with further work to clarify, include viral adaptation to culture environment or unequal distribution of virus exposed enteroids during experimentation. There is at least one report of serial passaging experiments using TMEV-DA and GDVII from 1976 to adapt virus to various BHK-21 or spinal or brain tissue-based cell cultures (Lehrich, Arnason et al. 1976). While this experiment had a single replicate and did not go further than 4-passages, this virus has an interesting history suggesting early manipulation for experimental purposes. Research on TMEV is almost exclusively done in cell culture or *in vivo* using intracerebral infections. Intracerebral infections are done for two main reasons: 1) biosafety because intracerebral infections of TMEV are not shed in the feces of infected mice (Modica, Sudyn et al. 2016), and 2) increased reproducibility of viral encephalopathies, seizures, and multiple sclerosis by-passing blood-brain barrier and immune responses (Dal Canto, Melvold et al. 1995).

This research demonstrated the need for additional research of TMEV infection in murine enteroids that include a much more global approach, such as RNA-Seq, various enteroid platforms or *in vivo* experiments, numerous research avenues exist to better understand the viral-host dynamic. TMEV is ubiquitous in biomedical research and used to recreate multiple sclerosis and certain types of epilepsy in murine models but there is a lack of research regarding its dissemination from the gut to neurological

tissue (DePaula-Silva, Hanak et al. 2017). Like other enteric viruses with known neurological dissemination (*i.e.,* poliovirus, EV71), there are known restrictions in the GI tract (Kuss, Etheredge et al. 2008) and understanding this gut-brain axis is paramount to understanding disease pathogenesis. While other enteroviruses have successfully been cultivated in human enteroids including poliovirus and enterovirus 71 (Drummond, Bolock et al. 2017, Good, Wells et al. 2019), our work with TMEV did not provide evidence of replication in murine enteroids. Interestingly, specific cell receptors for TMEV have not been identified but different cellular moieties for low neurovirulent and high neurovirulent have been identified as co-factors for TMEV attachment in neurological tissues (Lipton, Kumar et al. 2006). While this study did not specifically look for either sialic acid or proteoglycan heparan sulfate identified TMEV co-factors, literature suggests that these are readily produced in the murine intestinal tract and in intestinal enteroid cultures (Yamamoto, Nakase et al. 2013, Dubey, van Kerkhof et al. 2020).

To date this is the first report of TMEV exposure to murine intestinal enteroid cultures. While these results did not provide evidence for robust viral replication in this system, these results are valuable to researchers in the viral-host and TMEV communities providing information for future research inquires on viral infection of TMEV in the intestine. Future research in this area should include more global approaches to investigation including RNA-Seq to identify host immunological responses, use of knockout enteroid lines, and potentially *in vivo* confirmation.

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Scientific Contribution

Over the course of this dissertation, several different research paths were taken to better understand the fate, transport, disinfection, and viral-host dynamics of pathogens of public health importance and biomedical research. During the novel coronavirus pandemic, research related to the fate, transport, and disinfection of viral pathogens took on new importance where knowledge of surface survival and chemical disinfection became paramount in advising employers, businesses, and individuals how to reduce transmission of the novel coronavirus. Most of my work has focused on enteric pathogens, known for environmental transmission, high levels of excretion, and low infectious doses, however my work translated well to the growing needs of the pandemic and understanding surface disinfection and survival of enveloped respiratory viruses. This pivot in research was both necessitated by university restrictions and state stay-at-home orders and came at a time when viral-host-environment research on TMEV in intestinal enteroids indicated a more complicated disease dynamic that was outside our lab's environmental virology interests and capabilities.

With a world of uncertainty because of the pandemic, pivoting my research to encompass more of the disinfection and surface survival research was exciting and meaningful way to be helpful to apply skills and expertise I've honed over the course of my PhD program. We had several on-going disinfection projects that I had been working as side projects for our lab that I was able to continue working on and refocus my dissertation work. Much of this work involved surface survival and disinfection of viral surrogates, relying on bacteriophages MS2 as a surrogate for nonenveloped RNA viruses. We had demonstrated that HOCl was an effective disinfectant for MS2 on surfaces and could modify odor molecules that plaque sanitation facilities (Dennler-Church, Butz et al. 2020) hoping to provide simultaneous disinfection and improve malodors to encourage uptake of sanitation facilities. Expanding this work to include HOBr and pseudomonas bacteriophage phi6, an enveloped viral surrogate for coronavirus, helped to build evidence of survival and disinfection practices that would be effective for SARS-CoV-2 surface contamination.

In addition to expanding surface survival and disinfection of viral surrogates, our lab began surveilling wastewater from three separate wastewater treatment plants in King County testing seven different methods to detect SARS-CoV-2 and determine feasibility of wastewater surveillance on a weekly basis. This on-going study requires multi-day lab days, production of an internal viral control (human coronavirus OC43) and had expanded the capacity of all lab members. This important work contributed to the growing field of wastewater epidemiology and understanding risks associated with environmental transmission of SARS-CoV-2 (Philo, Keim et al. 2021). One nuance of this research was trying to understand the role of spiked internal control viruses and determining the best possible surrogate for validating SARS-CoV-2 detection methods across wastewater samples, internal controls, and qPCR methods to best describe the orders of magnitudes differences in recovery of spiked surrogate viruses that was being shown in the rapidly published literature (Chik, Glier et al. 2021). The question of internal control surrogate enveloped viruses in wastewater studies also demanded the same scrutiny for surface survival and disinfection studies of these same surrogates.

Prior to switching completely to surface survival and disinfection related SARS-CoV-2 research, I had been working on characterizing the viral-host-environmental dynamics of TMEV infection in murine intestinal enteroid cultures through a successful pilot grant that I wrote for. TMEV -as a murine Picornavirus- shares many physical, genetic, and disease characteristics with human poliovirus a common but close to eradication enteric viral pathogen. With poliovirus potentially being eradicated, scientific communities have been working to restrict its use in research settings (World Health Organization 2015) and phase it's use and storage out of labs unless they met biocontainment practices designed to minimize facility releases into susceptible populations. These new policies were a result of the planned eradication program necessary to phase out poliovirus work in research to prevent future outbreaks but the future of enteroviruses including several emerging non-polioviruses (EV-71, -68, coxsackieviruses) require continued research in this area. Therefore, it had been suggested that TMEV become an alternative enterovirus to human enteroviruses to be used in environmental microbiology experiments. Some Picornaviruses, including human poliovirus, are also known to have a viral-host interaction that is unique

in that it incorporates an intracellular antioxidant glutathione (GSH) into its protein capsid for thermal stability (Ma, Liu et al. 2014, Thibaut, van der Linden et al. 2014). As a protective mechanism, intracellular glutathione naturally fluctuates due to innate genetic differences (Chen, Xian et al. 2017) and environmental stressors including chemicals and metals (Ninkov, Popov Aleksandrov et al. 2015); we sought to characterize TMEV by its dependency on host GSH and which intestinal cells were important during infection. Initial results from experiments using cells with depleted GSH, found that TMEV was relatively resistance to fluctuations of intracellular GSH indicating that it was able to handle variable cellular redox environments (Appendix A: TMEV Sensitivity to Host Glutathione).

To investigate the host-environment relationship of TMEV, I developed a murine intestinal enteroid platform with variable GSH expression and replacement capacity of GSH from mice and were able to proliferate into multiple epithelial cells found in the intestine (Appendix B: Development of Murine Intestinal Enteroids with Variable GSH expression). These were partly validated and provided a platform for intestinal antioxidant toxicology and viral-host research. We also wanted to better understand TMEV infection in the GI tract. Even though this is a well-known viral pathogen in mice, there was a paucity of knowledge regarding its natural disease development where infection initiation begins in the GI and can eventually cause a range of neurological morbidities (DePaula-Silva, Hanak et al. 2017). As a first step in answering these scientific questions, we needed to expose murine enteroids to TMEV to verify viral replication and infection by observing increases in viral RNA through RT-qPCR. Our initial and subsequent studies indicated that there was no production in viral RNA and viral infection was not likely (Evaluation of Theiler's Murine Encephalitis Virus in Intestinal Enteroid Cultures). This was and continues to be one of the first studies that tried to determine and solidify enteroids as a potential TMEV research platform. While these initial results did not result in a robust replication, it did open research opportunities to ask why a typical GI virus did not appear to infect the epithelial cell represented and what host responses were restricting its infection. These research results can easily lead into larger research in the fields of cell biology, microbiome, immunology, and microbiology. With the pandemic

ramping up and the pilot grant ending, the need to pivot my efforts elsewhere means these research avenues remain unexplored.

Disinfection work has been a primary theme throughout the pandemic and in my work at UW. IN 2017 we began working with the WaPNRC to help them develop a wastewater disinfection protocol using off-label disinfectants to help mitigate environmental release and occupational exposure to recombinant viruses and viral vectors (Chemical Disinfection to Remove Viral Pathogens and Surrogates from Research Animal Wastewater to Address Occupational and Environmental Concerns). This research relationship did not follow the typical inquiry and investigation cycle of many research projects because this worked spanned over 5 years with disinfectants changing, and tested viruses being added or removed. This research was small-scale but had incredible meaning because of its collaboration and impact with the UW institutional biosafety committee. Based on the disinfectants chosen my research findings indicate that enveloped viruses are readily inactivated in wastewater, but non-enveloped viruses never experienced >3 $\log_{10}$ reduction values. A major component of this research was not the disinfection results but rather the lack of policy regarding containment for biohazardous waste for recombinant viruses and viral vectors. The *NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules* emphasize biosafety levels, risk groups, and protections but fall short in providing guidance on required $\log_{10}$ reduction of waste, level of sensitivity for detection, and monitoring. Labeling FDA and EPA registration for antiseptics and disinfectants have product requirements for microbial reductions for use but EH&S policies regarding biohazardous waste has only treatment recommendations. For biohazards such as recombinant nucleic acids and viral vectors, more investment in policy and guidelines for researchers are needed.

The other piece of my disinfection work was working with a local company Briotech. Briotech had developed a way to mass produce hypochlorous acid and was interested in expanding its application and conducting research. Through the help of Dr. Lori Robins at UW-Bothell, I was able to help develop protocols and run disinfection studies using HOCl and HOBr primary on MS2 bacteriophage. This disinfection work was paired with malodor work Lori's group was conducting and we were able to

publish this research (Dennler-Church, Butz et al. 2020) as an application to improve both sanitary conditions and alleviate malodors that are associated with community sanitation and pit latrines. Next, we wanted to do a side-by-side comparison of HOCl and HOBr disinfectants on MS2 because bromine is known to be more reactive than chlorine. This work found that HOBr was more effective than HOCl on MS2 leaving us with added questions about biological interactions, mechanisms, and individual protein modifications during these reactions (Direct comparison of the efficacy of hypohalous acids (HOCl & HOBr) in the inactivation of MS2 Bacteriophage as an Enteric Viral Surrogate). Additionally, Briotech and the NIH prion group is pursuing similar lines of research to better understand if this applies across pathogens.

Opportunities within the pandemic and throughout my dissertation were endless and gave me learning experiences that few doctoral students have in their tenures as students. Out of all the chaos, the accumulation of work helped to better characterize a murine picornavirus in relation to cellular redox status, and its ability to infect enteroid cultures with a hard transition into survival, persistence, and disinfection of viral pathogens and their surrogates. All these topics while leagues apart in topic, all fall within the scope of our department and contain elements of understanding health, our environment, and environmental transmission of infectious diseases.

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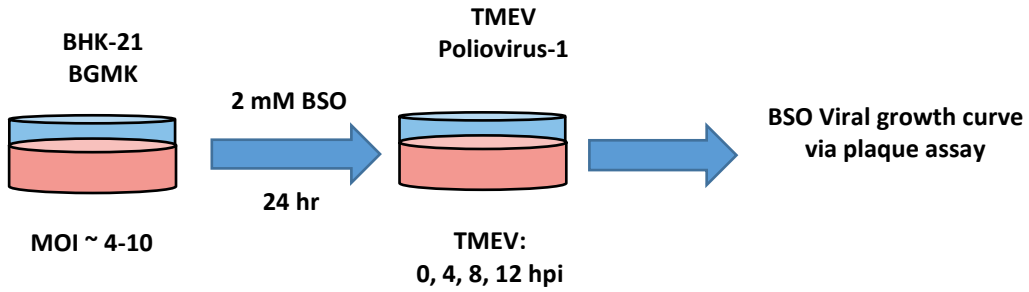
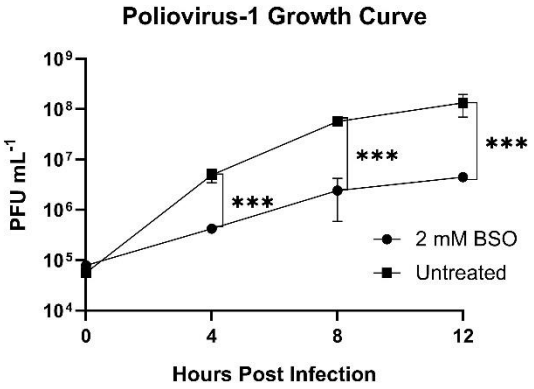
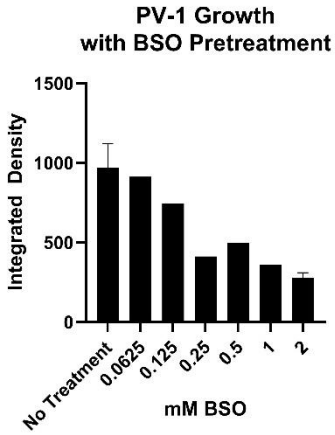
Appendix A: TMEV Sensitivity to Host Glutathione

Graphical Methods:



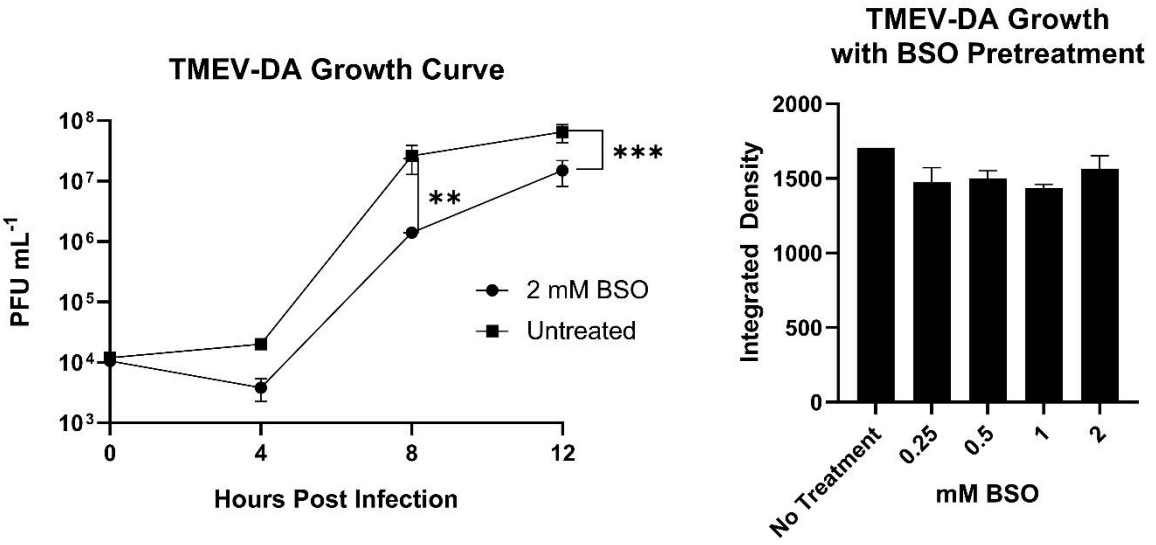
Poliovirus-1 Results:





BGMK cells were treated with or without 2mM BSO 24 h prior to infection with PV1. Cells and media was lysed and harvested at 0, 4, 8 and 12 h post infection and quantified via plaque assay. Statistically significant differences were found between treatment and control infections at 8 and 12 h by conducting multiple T-tests using the Bonferoni-Dunn Method, alpha=0.05

TMEV-DA Results:



BHK cells were treated with or without 2mM BSO 24 h prior to infection with TMEV-DA. Cells and media was lysed and harvested at 0, 4, 8 and 12 h post infection and quantified via plaque assay. Statistically significant differences were found between treatment and control infections at 8 and 12 h by conducting multiple T-tests using the Bonferoni-Dunn Method, alpha=0.05

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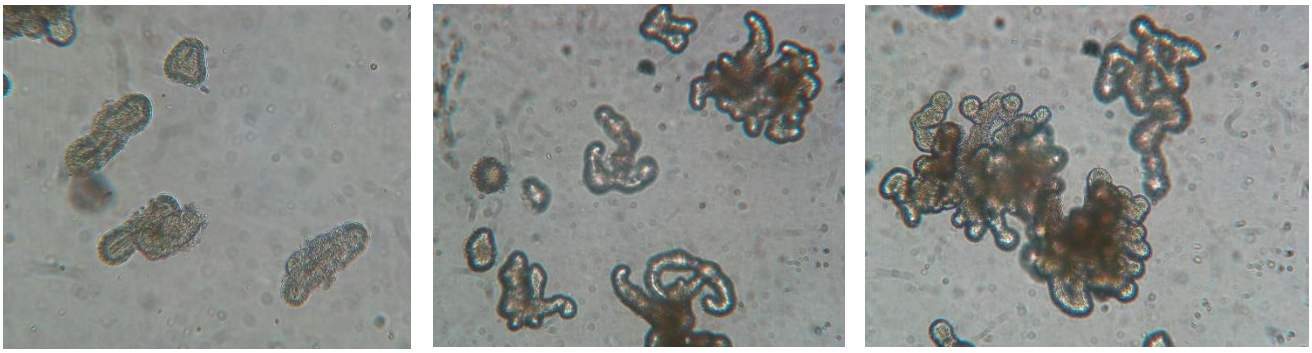
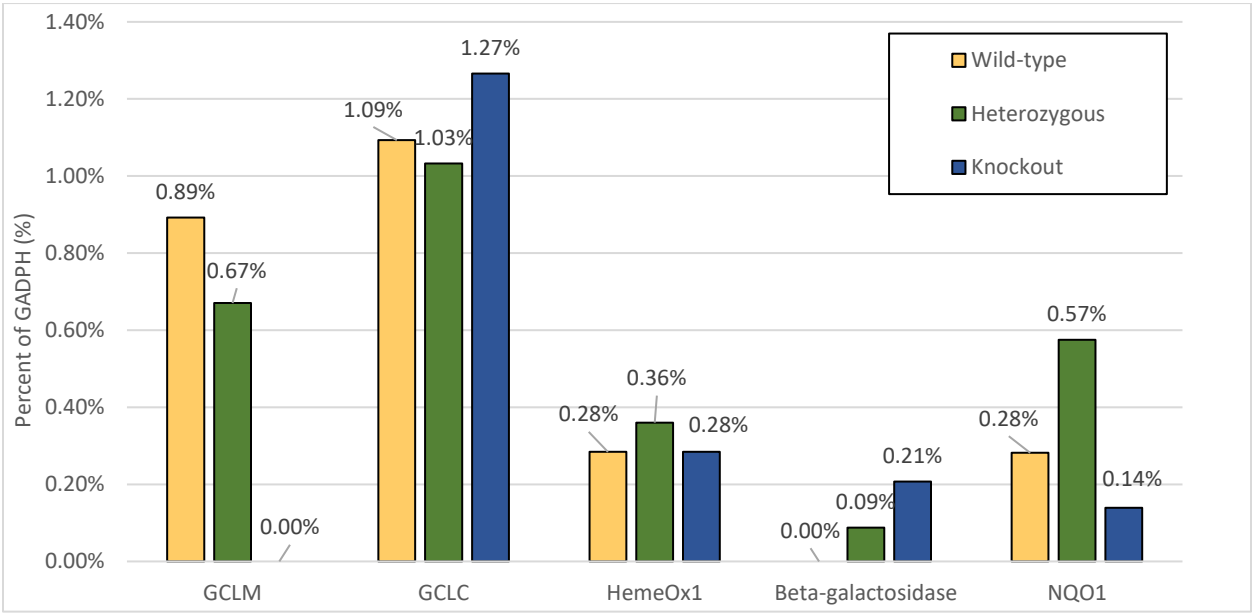
Thibaut, H. J., L. van der Linden, P. Jiang, B. Thys, M. D. Canela, L. Aguado, B. Rombaut, E. Wimmer, A. Paul, M. J. Perez-Perez, F. J. van Kuppeveld and J. Neyts (2014). "Binding of glutathione to enterovirus capsids is essential for virion morphogenesis." PLoS Pathog **10**(4): e1004039.

Appendix B: Development of Murine Intestinal Enteroids with Variable GSH expression



Day 1 Day 3 Day 5



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Appendix C: Immunofluorescence foci assay for TMEV, MAdV-2, and HCoV-OC43

Viral Fluorescence Plate Assay:

1. Seed cells into 96-well plates so that they are 80-90% confluent at the time of infection using 50ul -100 ul10% FBS, growth media per well.
    a. 96 wells= ~5 ml
    b. 48 wells= 2.5 ml

Infections:

1. Dilute samples serially by 3-fold in cell media, gen/kan + 2% FBS either using 1.5 ml tubes or in a separate 96-well plate
2. Remove cell growth media and add 100 µl/well of virus sample to cells and incubate virus in incubator and incubate cells until viral proteins are made:
    a. Poliovirus 6hr
    b. TMEV 6 hr
    c. MADv= 24-48 hrs/2 days
    d. HCoV-OC43 = 4 days

Fixing:

1. Immediately after growth period remove plate from incubator
2. Remove media and fix cells using 100 ul/ well 2% PFA diluted in PBS for 20 minutes at RT
3. Remove PFA and dispose of into PFA waste
4. Wash with PBS carefully 2x
5. Add 100 ul pf PBS per well

----- Plate can be stored at this point at 4˚ C until staining if wrapped in parafilm for a few days----------
Staining/Antibody Probes:

1. Remove PBS
2. Add 75 ul/well 20 mM Glycine/0.5% Triton X-100 in PBS.
3. Incubate for 20-60 min at RT
4. Remove and add 50 ul/ well **Primary Antibody** diluted in PBS + 0.05% Tween-80 + 1% BSA in PBS and incubate for 45-60 min at RT (See dilutions below)
    a. MAdV 8C4 anti-hexon- 1:900 diluted (Smith Lab) (Mouse)
    b. Anti-PV1 (Mouse) (1: 900)
    c. Anti-TMEV #473 (Rabbit) (1: 2000)
    d. Anti-TMEV H8-4 (Mouse) (1: 900)
5. Wash fixed cells 3X using PBS+ Tween
6. Add 50ul/ well of **secondary Alex fluor 488 antibody** or **secondary HRP antibody** diluted 1:1000 in PBST + 1% BSA and incubate for 45-60 minutes at RT
7. Wash fixed cells 2X using PBS+Tween (no BSA)
8. After last wash add 100ul/ well PBS
9. **Alexa 488**: Take to tox equipment room and Scan on Typhoon (520 bp40 w/ 488 laser)
10. **HRP antibody:** Add 1/10 diluted DAB solution (Thermo Scientific Pierce DAB substrate kit)

Optional:

- In place of step 8 of staining, add 50 ul of Topro-3, 1:2000 in PBST for 10 minutes at RT
- Wash 2x w/ PBS-T 5 minutes each
- After last wash add 100 ul PBS per well
- Scan on Typhoon (670 filter w/ 633 laser)

Primary Antibodies:

- MAdV 8C4 anti-hexon Monoclonal (mouse)
  - 1:900 diluted
  - 2ul in 1.8 ml PBS-TB (36 wells)
  - Aliquots of 4 ul into 3.6 ml of PBS-TB (72 wells)
- Anti-PV1 Monoclonal (Mouse)
  - 1:900 diluted
  - Aliquots of 4 ul into 3.6 ml of PBS-TB
    - 2ul into 1.8 ml PBS-TB
- Anti-TMEV #473 Polyclonal (Rabbit)
  - 1:2000 dilution 1.5 ul into 3ml
  - Aliquots of 2ul into 4ml PBS-TB
  - 1 ul into 2ml PBS-TB
- Anti-TMEV H8-4 monoclonal (Mouse)
  - 1:900 diluted
  - Aliquots of 4 ul into 3.6 ml of PBS-TB
    - 2ul into 1.8 ml PBS-TB
- Anti-HCoV nucleoprotein, Clone 542-7D monoclonal
  - 1: 900 diluted

Secondary Antibodies:

- Goat Anti-mouse Alex fluor 488 (3 ul) 1:1000 dilution
  - Add 3 ul antibody to 3 ml of PBS-TB for full 96 well plate
  - TMEV2, PV1 and MAdV antibodies
- Goat Anti-Rabbit Alex fluor 488 (3 ul) 1:1000 dilution
  - Add antibody to 3 ml of PBS-TB for full 96 well plate
- IgG (H+L) Cross absorbed Goat anti-mouse, HRP, Invitrogen
  - 1:1000 works for most samples

Other:

PBS/0.5% Tween-80

- 100 ml Gibco PBS
- 0.5 ml Tween-80
  - Mix, Autoclave

PBS/0.5% Tween-80 +BSA

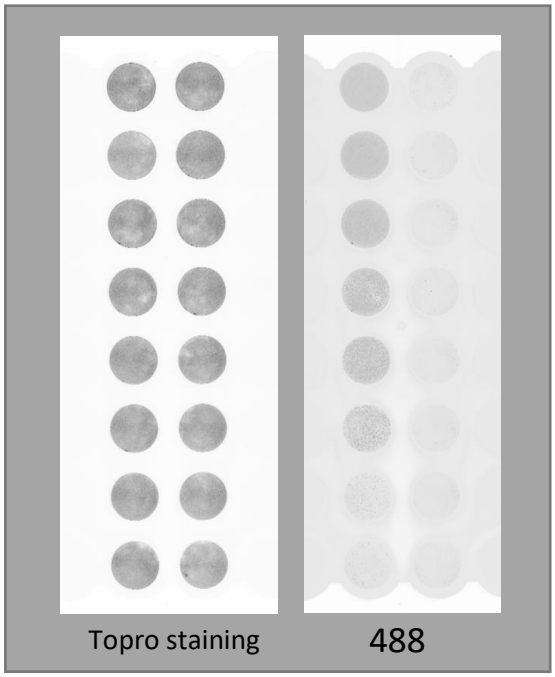
- 100 ml Gibco PBS
- 0.5 ml Tween-80
- 1 g BSA
  - Mix, keep in fridge use within week

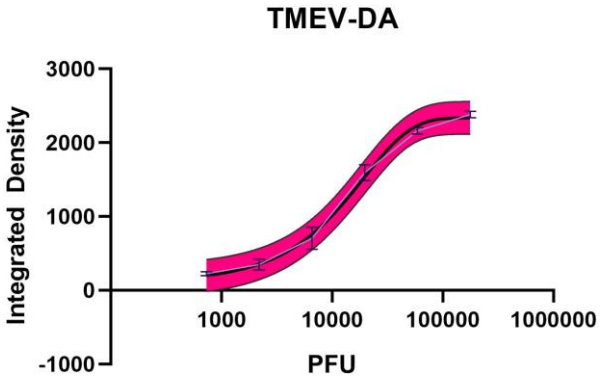
20 mM Glycine/0.5% Triton X-100:

- 400 ml Gibco PBS
- 0.6 g Glycine
- 2 ml Triton X-100
  - Mix thoroughly, autoclave

Machine: Typhoon 9400 variable mode imager

TMEV Typhoon imaging:





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Appendix D: EDGE Pilot Grant Proposal Submission (2018)

Title: Using a Novel 3D intestinal Tissue Culture System to Understand Modulating Effects of Host Glutathione under Environmental Cadmium Induced Oxidative Stress and Enteric Viral Infection

Status: Funded April 2018-2019

Abstract: Intestinal oxidative stress due to environmental cadmium (Cd) is mitigated by conjugation with the host-produced antioxidant, glutathione (GSH). GSH biosynthesis is controlled through gene transcription and the rate-limiting enzyme glutamate cysteine ligase (GCL); however, GCL genetic polymorphisms can reduce GSH production and capacity to mitigate intracellular oxidative stress. Viruses are dependent on host cellular function and metabolism where viral protein stability of some enteroviruses are dependent on sufficient levels of intracellular GSH. Therefore, it is essential to understand how host production of GSH and Cd, a broad oxidative stressor, affects both GSH-dependent and independent viruses in the intestine. To do this, we are proposing to use an intestinal stem cell derived murine enteroid tissue culture system with varied levels of GSH production (*gclm* -/-, -/+, and wild type mice) to look at the effects of host GSH production on genome replication, titer, and capsid stability in Theiler's murine encephalitis virus (TMEV) and mouse adenovirus-2 (MAdV-2) infections. From there we will pre-expose tissue cultures to hormetic and GSH-depleting levels of Cd prior to infections with TMEV and MAdV-2 to determine the impacts of Cd-mediated GSH levels during viral infection. In addition to looking at GSH production using a fluorescence plate assay, targeted gene expression profiles using RT-qPCR will be used to look at intestinal gene markers and host compensatory stress pathways. Viral genome replication, titer, and protein capsid stability will be monitored using qPCR/RT-qPCR, plaque assays, and thermal treatments. This interdisciplinary project will provide valuable information on the role of host oxidative stress as it relates to host GSH production with a complex exposure scenario. In line with the EDGE Center's mission, this study is one of the first to look at a gene-environment interaction in order to understand disease mechanisms between environmental contaminants, host gene regulation, and enteric viral illness.

Introduction:
The gastrointestinal (GI) tract is the first site where ingested contaminants are processed and absorbed into the body. Ingested environmental contaminants, such as heavy metals, are absorbed through the intestinal lining but often causes GI tissue and cellular injury, increasing susceptibility to further injury (Duizer, Gilde et al. 1999), reduced nutrient absorption (Chiocchetti, Velez et al. 2018), and infection (Kortman, Boleij et al. 2012). Tobacco smoke contains high levels of the toxic heavy metal cadmium (Cd), and cigarette smokers have high burdens of Cd in their tissues (Richter, Faroon et al. 2017). Excluding smokers, diet is the predominant exposure route for Cd in the general population through contaminated foods, with staple foods (grains, rice, cereals) having some of the highest Cd concentrations (Satarug, Vesey et al. 2017). Cd is also associated with certain occupational exposures leading to lung and kidney cancers; however, due to declining blood Cd levels over the last 30 years (Jarup and Akesson), research on lower dose exposures is imperative. Research on health effects due to Cd exposure has shown that current threshold levels are not protective enough (Satarug, Vesey et al. 2017) and that reevaluating the potential effects of diet based Cd on the intestinal epithelium is necessary.
As a first step in Cd detoxification, absorbed Cd conjugates with the thiol antioxidant glutathione (GSH) (Liu, Qu et al. 2009). At high Cd levels, GSH is completely depleted, allowing for the buildup of reactive oxygen in the cell, altering redox homeostasis and causing cellular oxidative stress (Hayes and McLellan 1999). In hormetic concentrations, low levels of Cd may enhance the biosynthesis of GSH aiding in cellular redox recovery. As one of the most important antioxidants, GSH biosynthesis is tightly controlled through the Nrf2-activation pathway (Ma 2013) and transcription of the rate limiting enzyme glutamate cysteine ligase (GCL) (Franklin, Backos et al. 2009). GCL is composed of two subunits; the larger catalytic subunit (GCLC) carries out catalysis; the smaller modifier subunit (GCLM) alters the catalytic properties of the GCLC. Genetic polymorphisms in *GCLC* and *GCLM* can lead to impaired production of GSH under oxidative stress, and are common in the human population (Garte, Gaspari et al. 2001, Singh, Lata et al. 2017) and have been associated with a number of chronic illnesses(Robertson, Harmon et al. 2003) and infectious diseases (Parsons, Campa et al. 2013). Increased oxidative stress due to polymorphisms in GSH production *could potentially* lead to altered viral infection in the intestinal epithelium, modifying disease outcome.

Production of intracellular GSH is both detrimental and necessary for viruses. Viruses are adapted to their host environment, taking over cellular machinery and metabolism for replication and transmission to other hosts. Increased production of GSH is beneficial for some- but not all- enterovirus infections by helping to stabilize the capsid. However, depletion of GSH produces unstable virions resulting in reduced viral fitness. Interestingly, GSH has no effect on enterovirus genome replication (Ma, Liu et al. 2014, Thibaut, van der Linden et al. 2014, Abdelnabi, Delang et al. 2017). Other viruses such as HIV and enterovirus-71 replicate better and enhance oxidative stress under compromised cellular states, likely by depleting GSH (Ho, Cheng et al. 2008, Parsons, Campa et al. 2013). To date, enteroviruses that have been shown to be GSH-dependent have been investigated only in human hosts. Theiler's mouse encephalitis virus (TMEV) is a closely related murine virus that is promising as a GSH-dependent model virus

because of its close phylogenetic relation to other human GSH-dependent viruses and for its infection in the murine intestinal tract (Feltz, Mandel et al. 1953, Tsunoda, Libbey et al. 2009). **In contrast**, adenovirus is an enteric DNA virus that also replicates in intestinal tissue but has a completely different replication strategy (not in the cytosol) and protein capsid composition (no disulfide bonds) from that of TMEV and enteroviruses, making it less likely to be affected by host GSH production. However, little is known about how Cd-induced host oxidative stress would affect the adenoviral life cycle. For GSH-dependent viruses and GSH-independent viruses alike, **investigating how host production of GSH and exposure to the oxidative stressor Cd is essential for understanding disease mechanisms that link environmental contaminants, host gene regulation, and enteric viral illness.**

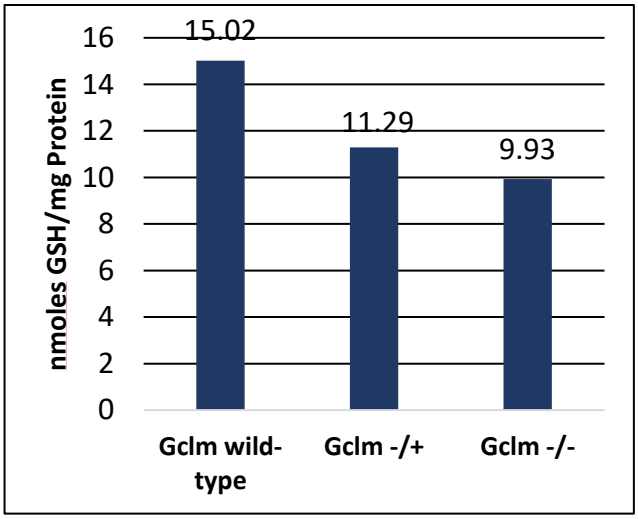

Figure 1: Preliminary data on differential levels of intracellular GSH in enteroid cultures at 5 days post passage using NDA fluorescence GSH detection assay (White, Viernes et al. 2003). n=1

Several research groups have demonstrated the utility of a differentiated intestinal enteroid model in disease mechanism research through successful replication of recalcitrant viruses (e.g. norovirus and rotavirus) (Saxena, Blutt et al. 2015, Ettayebi, Crawford et al. 2016, Drummond, Bolock et al. 2017, Wilson, Bromme et al. 2017). Beyond cultivation of recalcitrant viruses, enteroid cultures are advantageous as a research tool because they are more physiologically accurate than immortalized cell lines (Sato, Vries et al. 2009) (e.g. Caco-2 cells), allowing for comparison of disease mechanisms across different environmental exposures, where immortalized cell lines would not fully recapitulate host response. Adopting this method, our lab has recently established a glutamate cysteine ligase modifier subunit (GCLM) subunit knockout (-/-) and heterozygous (+/-) murine enteroid tissue culture system that has reduced *Gclm* mRNA expression and lower GSH levels (Figure 1) (McConnachie, Mohar et al. 2007) in order to study the role that oxidative stress has on enteric viral illness. Understanding differential GSH production and oxidative stress induced by environmental stressors (e.g. Cd) on viral infections is important for understanding gene-environment mechanisms that drive viral infections. Using TMEV and MAdV-2, **we hypothesize that hormetic Cd modulation of cellular GSH will directly correlate with viral fitness measures (e.g. genome replication, viral titer, and capsid stability) for RNA but not DNA viruses.** We will address this hypothesis through the following specific aims:

Aim 1: Demonstrate variation in virus genome replication, titer, and capsid stability as related to baseline intracellular production of GSH in mouse enteroid tissue culture systems using MAdV-2 and TMEV.

> **Hypothesis 1:** TMEV infections in *Gclm* knockout (-/-) and *Gclm* heterozygous (+/-) enteroid tissue culture will produce instable viral particles compared to *Glcm* wild-type enteroid tissue cultures.
>
> **Hypothesis 2:** MAdV-2 infections in murine enteroid tissue cultures of all genotypes will be comparable with regard to genome replication, titer, and capsid stability.

Aim 2: Determine the impacts of Cd-modulated GSH levels on viral fitness measures across hormetic and depleting dose levels for MAdV-2 and TMEV infections.

> **Hypothesis 2:** Cd induced oxidative stress will reduce TMEV titer in wild-type mouse enteroid cultures and create thermally unstable viral particles in a dose-dependent manner.
>
> **Hypothesis 2.1**: Cd induced oxidative stress will have no effect on MAdV-2 replication, titer, and capsid stability in all mouse enteroid cultures genotypes.
>
> **Alternative Hypothesis**: Cd induced oxidative stress will reduce replication, titer, and capsid stability of both TMEV and MAdV-2 in a dose-dependent manner due to antiviral properties of Cd in tissue culture.

Approach:
We have successfully cultured differentiated mouse enteroids from intestinal tissues of *Gclm* -/-, *Gclm* -/+, and wild-type C57BL/6 mice (McConnachie, Mohar et al.). They are grown in a 3D laminin scaffolded matrix (Matrigel) and overlaid with medium containing growth factors and hormones to promote crypt proliferation and cell differentiation (Sato, Vries et al. 2009). 3D cultures can then be harvested and plated into 96-well plates for apical access to polarized intestinal epithelium necessary for Cd introduction and viral infections. Currently, intestinal tissues are collected as incidental tissue from humanely euthanized mice under an approved IACUC protocol (PROTO201600672) with anticipated IACUC approval pending project funding.

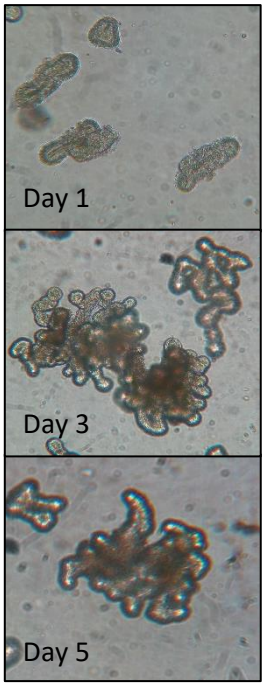

Figure 2: Bright field microscope pictures of expanding and differentiating gclm wild-type enteroids at 1, 3, and 5 days post passage.

As part of Aim 1, **we will challenge all three genotypes of enteroids with TMEV or MAdV-2**. Infectivity, replication, and titer will be examined using plaque assay and qPCR or RT-qPCR for viral genomes. Viral capsid stability, which is a defining aspect of viral GSH dependency, will be evaluated on post-infection viral progeny using an anti-GSH antigen pull down method (Ayene, Biaglow et al. 2008) and heat inactivation treatment with and without the presence of GSH and evaluated using plaque assays (Ma, Liu et al. 2014). Data from Aim 1 experiments will help elucidate variation in viral infection and viral capsid stability due to host GSH production in both TMEV and MAdV-2 infections.
To address Aim 2, all genotypes of enteroid tissue cultures will be grown and pre-conditioned in media containing increasing doses of $CdCl_2$. Based on GSH production and gene expression in *Gclm* enteroid genotypes, Cd doses that induce and deplete intracellular GSH will be used for viral infection studies. Enteroids cultured in inducible, depleting, and no Cd will then be infected with TMEV or MAdV-2 to determine the effects of Cd as a broad oxidative stressor. GSH levels will be monitored using a fluorescence assay (White, Viernes et al.), gene expression will be monitored by RT-qPCR for *Gclm* and *Gclc*, and Cd uptake will be measured using inductively-coupled plasma mass spectrometry.
Targeted gene expression profiling of enteroid cultures during Aims 1&2 of genes associated with stress pathways, divalent metal transporter-1, and intestinal gene markers will help to elucidate the synergistic relationship between host GSH production, enteric viral infection, and Cd oxidative stress. This will be conducted in the Functional Genomics, Proteomics and Metabolomics Facility Core at the EDGE Center using RT-qPCR. Data from targeted gene profiles would help to identify compensatory stress pathways in heterozygous and knockout

enteroid tissue culture. Targeted gene profiling would also help to establish alterations in intestinal cell differentiation features, intestinal tissue function, and altered cellular uptake of Cd into cells through divalent metal transporters because of reduced GSH production, Cd exposure, or enteric viral exposure.
One potential pitfall of the proposed research is the reliance of TMEV as a GSH-dependent virus. While TMEV does represent the most appropriate murine virus, it might not be GSH-dependent. As part of our research strategy we have multiple layers of verifying GSH-dependence using both plaque assays, GSH antigen pull down methods, and thermal challenge methods (Thibaut, van der Linden et al. 2014). Alternatively, Coxsackie B viruses are GSH-dependent human enterovirus that could replace TMEV if murine cells are infected at a high MOI.

Significance:

This project represents one of the first studies to look at host GSH production on the potentially synergistic relationship between an environmental contaminant (e.g. Cd) and viral pathogens. Using a novel intestinal tissue culture system with differential production of GSH, this project will inform the effects of Cd on intestinal epithelium, enteric viral infection mechanisms, and host glutathione response. Due to the interdisciplinary nature of this project, the proposed collaborative project between Dr. Jason Smith (Department of Microbiology), Dr. Terrance Kavanagh (DEOHS), and Dr. John Scott Meschke (DEOHS) is necessary for the completion of the proposed study. Additionally, in alignment with the EDGE Center and NIEHS missions, this grant would fund a project studying gene-environment interactions informing both animal and human health research. Finally, this project supports the EDGE training mission in providing funds to support an undergraduate researcher and project funds for a doctoral student's dissertation research.

Facility Core/Technology Usage:

This project plans to have gene expression profiles conducted in the Functional Genomics, Proteomics and Metabolomics Facility Core. Additionally, we have discussed with Dr. Edward Kelly, Director of the EDGE IEHSFC Microphysioloigcal System Unit, and member of the Hepatic, Renal and Gastrointestinal Collaborative research team on further developing our genetically altered intestinal enteroids as a research tool.

Collaborative Agreements:

Completion of this project would rely on the collaboration between Dr. John Scott Meschke, Dr. Terrance Kavanagh, and Dr. Jason Smith (Department of Microbiology). Each are uniquely qualified to support the completion of different parts of this project. Dr. Jason Smith has conducted research using both murine and human intestinal enteroid cultures to study innate immune viral responses. Dr. John Scott Meschke has research experience in environmental enteric viral illnesses. Dr. Terrance Kavanagh is a researcher in oxidative stress, glutathione biosynthesis, and creator of the *Glcm* knockout mouse line.

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Appendix E: IACUC Protocol 4432-01: Glutathione Production in Host-Virus Interaction

Basic Information

1. * **Select research team:**
   Meschke Microbiology Lab

2. * **Select admin office:**
   Default

3. * **Title of protocol:**
   Monitoring Glutathione production in Host-Virus Interactions

4. * **Short title:**
   4432-01: Glutathione Production in Host-Virus interaction

5. * **Summary of research:**
   This research is interested in how cellular oxidative stress, marked by a reduction in an antioxidant called glutathione, affects both the host and virus during enteric viral infection. We aim to determine if oxidative stress affects viral replication/infectivity, and host recovery/viability. To do this we are looking at one of the most common cellular antioxidants, glutathione, and its role in modulating enteric viral infection in experiments using murine primary gastrointestinal epithelial cell cultures.

   Causes of oxidative stress are widespread but environmental contaminants as oxidative stressor are of concern because health affects due to oxidative stress have been implicated in numerous chronic health effects. Oxidative stress as it relates to gastrointestinal illnesses is relatively new because there have not been appropriate tissue culture models for studying these viruses. However, more recently a tissue culture model using organ-like intestinal epithelial stem cell tissue culture systems have been introduced that are appropriate for replicating and studying these viruses, prompting interest in our research line looking at glutathione-host-virus interactions.

4432-01: Glutathione Production in Host-Virus interaction

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6. * Principal investigator:

John Scott Meschke

7. * What is the intention of the animal protocol?

Experimental Research

View: SF: Experimental Research Protocol Addition

Experimental Research Protocol Addition

1. * Will the protocol include breeding?

○ Yes ● **No**

4432-01: Glutathione Production in Host-Virus interaction

View: SF: Protocol Team Members

Protocol Team Members

1. **Identify each additional person involved in the design, conduct, or reporting of the research:**

Name	Role	Involved in Animal Handling	Authorized To Order Animals	E-mail	Phone
Terrance J Kavanagh	Co-Investigator	yes	yes	tjkav@uw.edu	+1 206 685-8479
Erika K Keim	Graduate Student	yes	yes	ekeim@uw.edu	

2. **If veterinary care will be provided by individuals outside of DCM or WaNPRC, provide the name, credentials, and contact information below:**

   N/A

4432-01: Glutathione Production in Host-Virus interaction

View: SF: Funding Sources

Funding Sources

1. Identify each organization supplying funding for the protocol:

	Funding Organization	Sponsor's Funding ID	Grants Office ID	Documents
View	National Institute of Environmental Health and Science (NIEHS)		A132069	

4432-01: Glutathione Production in Host-Virus interaction

View: SF: Scientific Aims

4432-01: Glutathione Production in Host-Virus interaction

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Scientific Aims

1. * Scientific aims of the research:

Aim 1: Demonstrate variation in virus genome replication, titer, and capsid stability as related to baseline intracellular production of GSH in mouse enteroid tissue culture systems using MAdV-2 and TMEV.

Hypothesis 1: TMEV infections in Gclm knockout (-/-) and Gclm heterozygous (+/-) enteroid tissue culture will produce instable viral particles compared to Glcm wild-type enteroid tissue cultures.

Hypothesis 2: MAdV-2 infections in murine enteroid tissue cultures of all genotypes will be comparable with regard to genome replication, titer, and capsid stability.

Aim 2: Determine the impacts of Cd-modulated GSH levels on viral fitness measures across hormetic and depleting dose levels for MAdV-2 and TMEV infections.

Hypothesis 2: Cd induced oxidative stress will reduce TMEV titer in wild-type mouse enteroid cultures and create thermally unstable viral particles in a dose-dependent manner.

Hypothesis 2.1: Cd induced oxidative stress will have no effect on MAdV-2 replication, titer, and capsid stability in all mouse enteroid cultures genotypes.

Alternative Hypothesis: Cd induced oxidative stress will reduce replication, titer, and capsid stability of both TMEV and MAdV-2 in a dose-dependent manner due to antiviral properties of Cd in tissue culture.

2. * Using language understandable to non-scientists, describe the goals and significance of the protocol to humans, animals, and science:

This research is hoping to better understand how cellular oxidative stress modulates the interaction between enteric viruses and susceptible host tissues. We are primarily interested in investigating the role of oxidative stress in adenovirus and Theiler's Murine Encephalitis Virus because of their high tropism in the gastrointestinal tract and for their high public health significance in GI infection in humans.

To do this we are looking at one of the most common cellular antioxidants, glutathione,

4432-01: Glutathione Production in Host-Virus interaction

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and its role in modulating enter c viral infection in experiments using primary gastrointestinal epithelial cell cultures. Using mouse models with varied levels of glutathione production, we will observe viral-host interactions in in vitro primary gastrointestinal epithelial stem cells challenged with enteric viral infection and oxidative stress conditions. In addition to using mice models with varied levels of glutathione production, we also want to test the modulating effects of a common environmental oxidative stressor, such as the heavy metal cadmium, as a pre-exposure to viral infection. This will allow us to determine if oxidative stress affects viral replication/infectivity, and host recovery/viability.

Information gained from the animal research in this protocol will directly aid in similar experiments planned for in establishing and using human gastrointestinal epithelial stem cells to learn more about the role of oxidative stress in gastrointestinal viral infections in humans to better understand disease mechanisms and inform public health.

At a larger scale, knowledge from this research can help elicit important disease mechanisms related to enteric pathogens to aid in the development of more effective therapies, identification of higher risk populations (i.e. populations with genetic polymorphisms), or important exposure scenarios related to enteric illness outcomes (i.e. dietary patterns).

4432-01: Glutathione Production in Host-Virus interaction

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3. * Provide a statement to address the potential harm to the animals on this study (e.g., pain, distress, morbidity, mortality) relative to the benefits to be gained by performing the proposed work:

Pain, distress, and mortality in mice due to this research will primarily be related to handling and intraperitoneal injection necessary to euthanize and subsequently harvest murine intestinal tissue necessary to establish murine tissue culture systems.

The knowledge gained from experiments in these in vitro methods greatly outweighs effects that might occur, because this research elicits valuable knowledge on the role of oxidative stress in enteric viral infections. Additionally, we believe that using our proposed methods of growing and expanding these primary intestinal tissue cultures from intestinal crypts reduces the number of animals necessary to conduct this research and could pave the way for future toxicological and virology studies.

001. Harvesting Intestinal Epithelial Crypts - Justify the need for any animals in pain category E: NA

i View: SF: Experiments

4432-01: Glutathione Production n Host-Virus interaction

Experiments

1. * Define the experiments to be used in this protocol:

Name	Species	Is USDA	Total	Pain Category	Common Procedures	Variable Procedures	Variable Description
001. Harvesting Intestinal Epithelial Crypts	Mice	no	108	N/A	• CO2 Followed by Cervical Dislocation (>10 days of age) (Standard - Euthanasia) [Archived]		N/A

2. If the experiments include survival surgery, will any single animal undergo more than one survival surgery? (include any animal that underwent surgery prior to use on this protocol)

○ Yes ● **No**

- **If you will be using animals to train personnel or to practice procedures included in this protocol, describe below and justify number of animals to be used for training:**

  N/A

4432-01: Glutathione Production n Host-Virus interaction

View: Cr ate and Edit

1. * **Experiment name:**
   001. Harvesting Intestinal Epithelial Crypts
2. * **Species:** (if Other species is selected, type name of species at beginning of Question 3)
   Mice
3. * **Describe the animal experience in the experiment, from enrollment in the study to the final endpoint, including all procedures in chronological order and the minimum time between procedures. Use "Supporting documents" to attach a related timeline, table, or flow chart:**

   1. Mice will be euthanized by CO2 followed by cervical dislocation.

   2. The small intestine will then be harvested for immediate processing for crypt isolation.

b. Type "N/A" in this field:
N/A

4. Scientific goal of this experiment:

The overall goal of this is to harvest small intestinal epithelial stem cells which can then be established into primary tissue cultures for further experiments on oxidative stress, viral infection, and oxidative environmental contaminants. These organoids, 'mini-guts', or enteroids, are made up of adult murine intestinal stem cells which have the ability to differential and renew intestinal tissue lining. These tissue culture systems can be utilized in a variety of viral infection, oxidative stress, and environmental exposure experiments.

While these cultures can be cryo-preserved, revived, and grown over a long period of time many of the cell functions wane over time necessitating the creation of new enteroids from mice periodically.

5. Select experimental procedures:

Name	Type	Version	Scope
CO2 Followed by Cervical Dislocation (>10 days of age)	Euthanasia	1	Standard

6. Do not add procedures.

a. Section for OAW use only.

Name	Type	Version	Scope
There are no items to display			

7. Type "N/A" in this field:

N/A

8. Type "N/A" in this field:

N/A

9. * Total number of animals used in this experiment: (including all the animals to be produced)

108

10. * Identify husbandry exceptions:

	Exception Type	Description and Justification
View	Mice - No husbandry or enrichment exceptions.	

11. Supporting documents:

Document Name	Date Modified
Crypt Isolation Protocol(0.01)	4/17/2018 12:11 PM
Sample Size calculation(0.01)	4/6/2018 10:02 AM

12. Indicate the animal sex (female, male, both), animal ages, and animal size:

Age: 5-15 weeks

Sex: Male, Female

13. Describe the humane endpoints (criteria for euthanasia) and the monitoring protocol, including frequency and specific behavioral and clinical signs to be monitored:

Monitoring protocol: Post- transfer of mice from the other IACUC protocol mice will be monitored daily for 1-3 days prior to euthanasia for tissue harvesting by vivarium staff and lab personnel.

Behavioral and clinical signs: any signs of lethargy, huddling, unkempt fur or evidence of illness.

Care and treatment: If clinical s gns indicate animal is adversely affected th y will be euthanized and disposed of. Consultation with DCM veterinary staff will help determine treatment or euthanasia decisions.

14. Will some animals live out their natural lifespan as part of this experiment? If so, indicate their use and describe the monitoring plan for aged animals (e.g., rodents > 18 months of age), including frequency, behavioral and clinical signs to be monitored, and criteria for euthanasia:

No

15. If there is expected mortality (spontaneous death) in this experiment:

a. Procedure/condition associated with mortality:

There is no spontaneous death expected during these procedures.

b. Estimated mortality rate, i.e. percentage of animals expected to die spontaneously (not via euthanasia) or need to be euthanized as a result of the procedure. (Be sure to account for this in your animal number calculations):

NA

c. Explain why euthanasia is not possible or appropriate:

NA

16. Justify the total number of animals used in this experiment: (i.e., the number listed in Question 9)

Main variables that are important for developing tissue cultures to study oxidative stress in tissue are related to: glutamate cysteine ligase modifier (gclm) subunit status, and animal sex. Gclm status can be divided up into 3 different genotypes: null, heterozygous, and wild-type. Both heterozygous and wild genotypes produce roughly the same levels of intracellular glutathione while null genotypes produce around 23% of the same levels of glutathione compared to the wild type. Animal sex has also been shown to be a factor in oxidative stress with male mice showing diminished levels of GSH levels in liver, kidney, and brain compared to females (Data provided by Kavanagh lab).

Since the experiment has been designed with the intent to collect tissue and establish long living intestinal tissue culture systems, called enteriods, derived from mice, we plan on having 6 distinct enteroid experimental groups on the basis of animal genotype and sex. These enteroids are able to be grow in media for longer periods of time but cellular functions start to diminish over time (information provided by Smith lab) and must be replaced regularly.

Using 6 experimental groups based off our main variables (gclm status/genotype and animal sex) and the OAR provided sample size calculator we would need 6 replicates for each treatment trial (see attached sample size calculation). We believe that having two animals in each group will be sufficient for intestinal crypt isolation and observation and that over the course of year these enteroid cultures would need to be replaced 3 times per year.

We expect the total number of animals needed over the next three years for this experiment to be 108 mice.

View: Custom: Cr ate and Edit

1. * Exception type:

Mice - No husbandry or enrichment exceptions.

2. Description and justification:

Procedure Personnel Assignment

1. Select the team members who will be performing each procedure:

Procedure	Species	Is USDA Species	Team Members
Euthanasia: CO2 Followed by Cervical Dislocation (>10 days of age), ver. 1 (Standard) [Archived]	Mice	no	John Scott Meschke Terrance J Kavanagh Erika K Keim

2. Team member training:

Filter by First Name

Enter text to search Clear All

One or more data sources failed to load. Contact your system administrator for assistance.

First Name	Last Name	Training

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n

Strains

1. Identify strain/stock for rodents and genetically modified animals, if applicable:

	Species	Is USDA Species	Strain	Genetically Modified Strain	Phenotype
View	Mice	no	Gclm heterozygouse (+/-)	yes	N/A
View	Mice	no	Gclm null (-/-)	no	N/A
View	Mice	no	Gclm wild type (+/+)	no	N/A

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View: SF: Animal Justification

Animal Justification

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n

1. Adjust the number of animals to be used or produced for this protocol as needed:

Species	USDA Covered Species	Pain Category	Animals Identified in Experiments	Adjusted Animal Count
Mice	no	Pain Category B	0	0
Mice	no	Pain Category C	108	108
Mice	no	Pain Category D	0	0
Mice	no	Pain Category E	0	0

2. If you adjusted the number of animals for this protocol, explain why:

N/A

3. * Provide the rationale for using animals in this protocol:

Mouse derived intestinal tissue cultures are some of the most appropriate ways to study the effects of environmental oxidative stressors and enteric viral infections without using whole animals as experimental units. These tissue culture systems can be derived from a single animal at a time and can be expanded and grown for up for a long period of time. These tissue cultures best recapitulate the intestinal epithelium and are the most appropriate for looking at both toxicological and virological responses.

4. * Type "N/A" in this field:

N/A

5. * Justify why each proposed species was chosen for this protocol:

Mouse derived intestinal tissue cultures are some of the most appropriate ways to study the effects of environmental oxidative stressors and enteric viral infections without using whole animals as experimental units. These tissue culture systems can be derived from a

single anima at a time and can be expanded and grown for up for a long period of time. These tissue cultures best recapitulate the intestinal epithelium and are the most appropriate for looking at both toxicological and virological responses.

6. Identify each source of animals for this protocol if not purchased through DCM or WaNPRC, transfers from other UW or non-UW protocols: (If transfers, see Question 9 below.)

Not purchasing through DCM or WaNPRC: N/A Transferred from another UW Protocol, provide Protocol Number: 2384-08 If animals were transferred, please provide an explanation of all procedures the animal(s) experienced prior to transfer (i.e substance administrations, surgeries, dietary restrictions, etc.): None

7. Supporting documents:

Document Name	Date Modified
There are no items to display	

8. If you are carrying over non-human primates (NHPs), please note the number of animals, as well as the associated experiment. Note: Include these animal numbers in your justification(s) in the associated experiments:

9. If animals are transferred, describe any procedures animals will experience prior to transfer, e.g., substance administration, surgeries, dietary restrictions, etc.:

None

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View: SF: Alternatives Searches and Duplication

Alternatives Searches and Duplication

1. Record all searches for alternatives for each procedure that causes pain or distress:

	Search Date	Searched Databases	Keywords
View	4/5/2018	EMBASE (searches multiple databases) Web of Science (searches multiple databases) PubMed/Medline	N/A

2. Briefly describe the results of your searches and why you can or cannot incorporate the findings. Or, if a literature search was not performed, describe the methods used to determine that alternatives are not available or feasible:

The majority of results yielded methods that we are currently proposing to use by developing long lived and self-replicating intestinal tissue culture systems.

Other alternative methods use the entire animal as an experimental unit- using more mice than currently proposed.

True alternatives that we found in the literature, suggest using human surgical tissue as an alternative to mouse intestinal tissue but would not be compatible with our proposed viruses.

Lastly, immortalized cancer cell lines, such as Caco-2 cells, have been used in similar toxicological and virology studies but have been shown to have altered metabolic systems with up-regulated oxidative stress pathways compared to non-cancer cell lines. Our study focuses on oxidative stress pathways and it is necessary that these pathways fully recapitulate pathways in healthy individuals to provide scientifically accurate conclusions.

We believe that our proposed methods are the most effective for both answering our scientific questions and reducing the number of animals necessary to address important toxicological and virology questions.

3. * The principal investigator asserts that the activities described in this proposal do not unnecessarily duplicate previous experiments:

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◯ Yes ◯ No

4. Describe how the three R's (refinement, replacement, and reduction) have been employed on this project. Include alternatives that were considered for any procedures that cause pain or distress:

*** Refinement:** (use of methods to decrease animals' sensitivity to pain)
We anticipate studying the modulating effects of glutathione production on both gastrointestinal viral infection and environmental cadmium stressors in tissue culture models, which do not require the use of live animals in order to develop.

In order to minimize the pain and suffering of any animals used to harvest intestinal tissue, they will first be humanely euthanized using an AVMA-approved method. This will ensure that any tissue harvested would be conducted on animals as humanely as possible.

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*** Replacement:** (include in vitro tests, use of less sentient animals)

Our proposed course of study uses mice to develop relatively long lasting tissue culture systems that can expanded in vitro and used in multiple experiments over 4 months prior to replacement. This would replace entire animals that would otherwise be used as single experimental units for similar studies and replacing entire animals with these in vitro tissue culture systems.

*** Reduction:** (use of fewer animals to attain statistical significance)

Our proposed tissue harvesting of murine intestinal crypts will allow our lab to grow and expand these cells - allowing us to conduct a number of experimental replicates over a long period of time. This allows us to reduce the total number of mice used, while retaining the appropriate number of statistically significant experimental replicates necessary to answer scientific questions of interest.

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View: SF: Housing and Use

Housing and Use

1. Identify each vivarium location where animals will be housed or used:

	Name	Species	Hours
View	Roosevelt ABSL1	Mice	Greater than 24 hours
View	Roosevelt ABSL1	Mice	Greater than 24 hours

2. Identify each non-vivarium location where animals will be housed or used:

Name	Species	Hours	Reason
There are no items to display			

4432-01: Glutathione Production in Host-Virus interaction

View: custom_Add Vivarium Loca

View: UW IACUC Select Vivarium Room Level

1. Identify the location where animals will be housed or used inside the vivarium:

Roosevelt ABSL1

If you cannot find the location above, identify it here:

2. * What species will be housed or used in this location?

Common Name	Scientific Name
Mice	Mus

3. * How many hours will the animals be kept in this location?

Greater than 24 hours

4. Describe how this location will be used:

Housing

5. Describe how animals will be transported to and from this location, including container and route: (Note: Use of private vehicles requires IACUC approval)

N/A

1. * Campus:

Vivarium

2. * Vivarium:

Roosevelt Vivarium

3. * BSL Level:

Roosevelt ABSL1

4432-01: Glutathione Production in Host-Virus interaction

View: custom_Add Vivarium Loca

View: UW IACUC Select Vivarium Room Level

1. **Identify the location where animals will be housed or used inside the vivarium:**
   Roosevelt ABSL1

   If you cannot find the location above, identify it here:

2. * **What species will be housed or used in this location?**

Common Name	Scientific Name
Mice	Mus

3. * **How many hours will the animals be kept in this location?**
   Greater than 24 hours
4. **Describe how this location will be used:**
   Location will be used for euthanasia, tissue harvest and collection of mice.
5. **Describe how animals will be transported to and from this location, including container and route:** (Note: Use of private vehicles requires IACUC approval)
   N/A

1. * **Campus:**
   Vivarium
2. * **Vivarium:**
   Roosevelt Vivarium
3. * **BSL Level:**
   Roosevelt ABSL1

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View. SF. Disposi

Disposition

1. **Disposition plans for the animals when this research is complete:**
   (check all that apply)
   Euthanasia

2. **If Other, provide an animal disposition description:**
   N/A

3. **If protocol involves fixing tissues, list agents (e.g., paraformaldehyde, formalin):**
   N/A

4432-01: Glutathione Production in Host-Virus interaction

View: SF: Supporting Documents

Supporting Documents

1. Attach supporting files:

Document Name	Date Modified
There are no items to display	

• CO2 Followed by Cervical Dislocation (>10 days of age) (Standard - Euthanasia) [Archived]

View: SF: Procedure Identification

Procedure Identification

1. * Name of the procedure or surgery:

CO2 Followed by Cervical Dislocation (>10 days of age)

2. * Select procedure type: Euthanasia

3. * Species:

Mice

4. * Admin office:

Default

5. * Will administering this procedure, or will the intended effects of this procedure, cause any more than momentary pain or distress in the absence of anesthesia/analgesia?

○ Yes ● **No**

If yes,

a. Identify expected symptoms from administering this procedure:

N/A

b. Identify criteria under which animals will be removed from research:

N/A

4432-01: Glutathione Production in Host-Virus interaction

• CO2 Followed by Cervical D slocati n (>10 days of age) (Standard - Euthanasia) [Archived]

View: SF: Euthanasia

Euthanasia

1. * **Method of euthanasia:** CO2 Overdose

2. **Describe procedure:**
   CO2 will be administered from a compressed commercial cylinder utilizing a flow meter to deliver 10-30% of the chamber volume/minute. Total gas exposure will be at least 5 minutes, with gas flow being maintained for at least 1 minute after apparent clinical death. A timer will be used to ensure adequate length of exposure.

   Cervical dislocation will service as the secondary method of euthanasia. This procedure will only be performed by certified protocol personnel.

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View: SF: Procedure Documents

3. Describe how death will be confirmed:

Death will be confirmed by lack of respirations and heartbeat.

4. If animals will be anesthetized prior to euthanasia, select the anesthesia procedures to be used:

There are no items to display

Type "N/A" in this field:

5. Is this method approved by the AVMA Guidelines on Euthanasia (2020)?

● Yes ○ No

a. If no, provide a scientific justification for its use:

N/A

• CO2 Followed by Cervical D slocati n (>10 days of age) (Standard - Euthanasia) [Archived]

Procedure Documents

1. Supporting documents:

Document Name	Date Modified
There are no items to display	