Abstract

TUNG, GRACE. Efficacy of Commonly Used Disinfectants for Inactivation of Human Noroviruses and its Surrogates. (Under the direction of Dr. Lee-Ann Jaykus).

Human noroviruses (HuNoV) are the leading cause of food borne disease. There is a need to better characterize the efficacy of standard disinfectants, and elucidate the relationship between virus infectivity and molecular detection for HuNoV and their cultivable surrogates. The purpose of this study was to compare the efficacy of three commonly used disinfectants against representative HuNoV strains and cultivable surrogates using quantitative reverse transcription PCR (RT-qPCR) and infectivity assay. Ethanol (50%, 70% and 90%), sodium hypochlorite (5, 75, 250, 500 and 1,000 ppm) and a quaternary ammonium compound (QUAT, at 0.1X, 1.0X, and 10X concentrations) were evaluated against two HuNoV genogroup II strains (GII.2 and GII.4) and two surrogates [feline calicivirus (FCV) and murine norovirus (MNV-1)]. Virucidal suspension assays (30-sec exposure) were conducted in accordance with ASTM E-1052. Virus inactivation was quantified using RT-qPCR targeting the *orfI-orfII* junction (HuNoV), the RNA polymerase region (MNV-1), or the *orf* I region (FCV); infectivity assays were also performed for MNV-1 and FCV. Log reductions of 0.6, 2.3 and 2.3 were observed by RT-qPCR for MNV-1 after 30 sec exposures to 50, 70 and 90% ethanol, respectively. By infectivity assay, these numbers were 0.3, 3.5 and >3.5. For FCV, HuNoV GII.2 and GII.4 strains, there was no statistically significant ($p<0.05$) reduction in virus titer, regardless of ethanol concentration, by either RT-qPCR or infectivity assay. By RT-qPCR, HuNoV GII.2 was completely resistant to chlorine

concentrations ≤1,000 ppm; GII.4 showed a 4.5 log reduction at 1,000 ppm chlorine but no significant inactivation at concentrations ≤500 ppm. MNV-1 and FCV were much more susceptible to chlorine, demonstrating a 3.0 log reduction at concentrations ranging from 250-500 ppm. Log reductions based on RT-pPCR and infectivity assay (MNV-1 and FCV) correlated well for chlorine treatments. The QUAT compound was ineffective against all four viruses ($<0.5 \log_{10}$ reduction) at all concentrations tested. This study demonstrates the relative ineffectiveness of disinfectants against HuNoV and highlights the fact that behavior of the cultivable surrogates does not always mimic that of human strains.

Efficacy of Commonly Used Disinfectants for Inactivation of Human Noroviruses and its Surrogates

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BIOGRAPHY

Grace Tung was born in Buffalo, New York to David and Hui-ling Tung. She spent most of her childhood growing up in Centerville, Ohio until her family moved to Michigan during her high school years. She graduated from Troy High School in 2004 and was an active participant in the school orchestra and tennis team. In August 2004, she began her undergraduate education at Michigan State University (MSU) in East Lansing, Michigan. In May 2008, Grace graduated with honors from MSU as a member of the Honors College with a Bachelor of Science degree in Lyman Briggs School-Microbiology and a specialization in Food Processing and Technology. In June 2008, she began pursuing a Masters of Science degree in Food Science at North Carolina State University under the direction of Dr. Lee-Ann Jaykus.

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CHAPTER ONE

LITERATURE REVIEW

1.1. INTRODUCTION

Food borne illness is a major cause of morbidity and mortality worldwide, with 9.4 million cases, about 56,000 hospitalizations, and 1,351 deaths caused by 31 known pathogens in the U.S. alone (96). When also taking into account cases associated with unspecified agents, the numbers are much higher, with a combined total of 47.8 million episodes per year (95). Similar to earlier reports by Mead et al. (1999), Scallan et al. (2011a, b) reiterate the importance of viruses, which are now estimated to cause 59% of food borne disease cases (81, 95, 96).

There are many viruses that can be transmitted by food borne routes, including human enteroviruses (poliovirus, coxsackievirus, and echovirus), enteric adenoviruses, hepatitis A and E viruses, parvovirus, rotavirus, noroviruses and sapoviruses, among others. However, from an epidemiological standpoint, the agents responsible for the majority of acute viral gastroenteritis are the human noroviruses (NoV); these viruses also cause most viral food borne illnesses of known etiology, constituting between 3.3 and 8.3 million cases per year (96). Even these figures may be considered an underestimate because the disease is notoriously under-reported and cases frequently go undetected or outbreaks uninvestigated.

Human NoV cause a disease characterized by vomiting (hallmark symptom), nausea, diarrhea, abdominal pain, and sometimes headache and low grade fever. The illness is usually self-limiting, but on occasion will require hospitalization to treat dehydration. By virtue of the sheer numbers of food borne human NoV cases per year, even a low likelihood of severe disease (0.03%) or death will still result in a large number of serious outcomes. Current estimates are that human NoVs are responsible for 15,000 hospitalizations and 1500 deaths annually in the U.S. (96). Similar incidence estimates are available for other industrial countries (including: England, Wales, Finland, Sweden, the Netherlands, and Germany) (71, 113).

The first human NoV was identified by electron microscopic examination of stool samples collected from a 1968 outbreak of acute non-bacterial gastroenteritis that occurred at an elementary school in Norwalk, Ohio (50). Since then, scientists have come to understand and appreciate the significance of these so-called "small round structured viruses". Specifically, the human NoV are members of the *Caliciviridae* family, a group of non-enveloped RNA viruses displaying characteristic cup-like surface morphology upon examination using electron microscopy. This family is comprised of four genera (*Vesivirus, Lagovirus, Norovirus* and *Sapovirus*, the latter two of which are of significance to humans). The *Norovirus* capsid consists of 180 identical protein molecules that self assemble to producing a capsid composed of two domains, the shell (S) and protruding (P) domains; the P domain is further divided into two sub domains (P1 and P2) (9). The human NoV genome is a single stranded, positive sense

RNA of 7.4-8.3 kb in length that consists of three open reading frames. ORF1 is the largest and encodes the nonstructural proteins (e.g., protease and the RNA dependent RNA polymerase); ORF2 encodes the viral capsid proteins; and ORF3 encodes a small basic protein of unknown function (23, 46). The viral RNA is attached to a protein called VPg, which is thought to be a primer for RNA synthesis and helps transport the genome for negative strand synthesis (35). The genome also contains a 5' CAP (a modified guanine nucleotide that promotes genome stability) and a 3' poly-A tail (29, 46).

There are currently more than forty different human NoV strains classified into five genogroups (GI-GV). Genogroups I and II are associated primarily with human infections and are represented by the prototype strains Norwalk virus and Snow Mountain virus, respectively (25, 29); the other genogroups are predominantly animal viruses(115). *Norovirus* genogroups can be further subdivided into genotypes on the basis of the RNA sequence of the capsid region. Viruses within the same genotype, also known as genoclusters, share 69-97% nucleotide sequence similarity (29). Genogroup I is currently divided into eight genoclusters, while GII has at least seventeen. Genogroup II viruses are responsible for about 73% of human NoV illnesses, while GI viruses cause 26% (115). Over the last decade, the epidemic GII.4 strain and its variants have been of particular interest, and these currently are responsible for >50% of all human NoV outbreaks worldwide (13).

Historically, the fecal-oral route has been considered the most common means by which human NoV are transmitted, although aerosol formation after projectile vomiting also may be important. Their infectious dose is quite low, and very high numbers (10^8 genome copies or more per gram) are shed in the stool of infected individuals (2, 105). In addition, up to 30 million virus particles can be released in an episode of vomiting (76). Immunity to human NoV is poorly understood and appears to be transient, so people continue to be susceptible to and may experience multiple infections over the course of a lifetime (67). For these reasons and others, human NoV are considered highly transmissible between people, with perhaps most outbreaks occurring in association with close contact of individuals sharing living spaces such as in the case for families, and in schools, hospitals and nursing/extended care facilities, daycare centers, and on cruise ships. Nonetheless, consumption of contaminated food or water, or direct contact with virus-contaminated surfaces (fomites), remain very important exposure routes.

1.2. THE CULTIVATION DILEMMA

A major barrier to better understanding of human NoV lies in the fact that humans are their sole host, and hence there are no relevant animal models, nor are there *in vitro* mammalian cell culture-adapted human strains. In the early days after their identification, human NoV could only be detected by electron microscopy. This changed around 1990 with the introduction of molecular amplification methods,

specifically reverse transcription polymerase chain reaction (RT-PCR). More recently, RT-PCR has been further improved by incorporating fluorescently labeled probes that capitalize on the fluorescence resonance energy transfer (FRET) phenomenon, allowing RNA amplification and confirmation (via a form of hybridization) to occur while the amplification is proceeding, hence the term real-time or quantitative RT-PCR (RT-qPCR).

Historically, it was assumed that RNA molecules are quite unstable owing to the near universal presence of RNases. However, over a decade ago, investigators reported that both 16S ribosomal RNA and messenger RNA from bacterial cells can persist after cell death (80). Soon thereafter, others reported that, depending on the sample matrix, the genome material from RNA viruses persists longer than does infectious virus. For example, Wetz et al. (2004) demonstrated that it took as long 28 days for complete degradation of poliovirus RNA suspended in seawater; similar data have been reported for other enteric viruses (21, 32, 99, 110), although a few studies have shown conflicting results (36). Nonetheless, there is general scientific consensus that viral RNA is stable long after its release from capsids, and hence, although a positive detection signal by RT-PCR provides evidence of viral RNA, it cannot assure that infectious virus is present. In other words, RT-PCR cannot reliably predict virus infectivity.

1.3. A ROLE FOR NOROVIRUS SURROGATES

Because human NoV cannot be cultivated *in vitro*, along with the poor correlation between molecular amplification and virus infectivity, investigators have relied on cultivable surrogate viruses as proxies. The most widely used surrogates are other viruses in the *Caliciviridea* family, specifically feline calicivirus (FCV) and murine norovirus (MNV-1) (98). The former belongs to the *Vesivirus* genus and is naturally occurring in feline populations, typically affecting the oral cavity and upper respiratory tract of cats (46, 91). First reported as a surrogate for human NoV by Slomka and Appleton (1998), FCV is particularly appealing because it is easy to culture and quantify, leading to its wide use in disinfection and inactivation studies (103). However, FCV belongs to a different genus and its genome varies significantly from that of the *Norovirus* genus. Further, FCV is transmitted by a respiratory rather than a fecal-oral route (33, 91). Perhaps most importantly, FCV is much more sensitive to extremes of pH than other surrogates and human NoV (14, 26, 106).

Other animal caliciviruses (e.g. enteric canine calicivirus) have been used as surrogates, but these also show elevated sensitivity to extremes of pH (<3 and >7) (31). Clearly a cultivable member of the *Norovirus* genus would be a more justifiable surrogate, and strains that infect a variety of species including cattle, swine and mice have been identified. To date only one animal NoV (murine norovirus, or MNV-1) has been cultivated *in vitro* (51, 114). Like human NoV, MNV-1 is transmitted by the fecal-oral route and the virus shares many biochemical and genetic features with human

strains, including size, shape and buoyant density (114). However, there are differences. For example, MNV-1 does not bind to the histoblood group antigens (HBGAs), purported host cell receptors or co-receptors for human NoV (114). Further, MNV-1 causes a lethal infection in mice that is manifested as hepatitis, pneumonia, or inflammation of the nervous system (51). Finally, MNV-1 appears to be more sensitive than FCV to dry conditions (14) and recent studies have shown that it is more sensitive to disinfectants than human NoV, at least in comparative RT-qPCR studies (39). Taken together, it is generally recognized that, while infectivity data for surrogates are useful, the behavior of the surrogates is not always representative of that of human strains. Hence, interpretation of results from surrogate experiments must be approached with caution.

1.4. ENVIRONMENTAL PERSISTENCE OF NOROVIRUS

Epidemiological evidence from prolonged outbreaks on cruise ships, hotels and hospitals suggests that human NoV are environmentally persistent (62, 69) and this undoubtedly contributes to their success as food borne disease agents. For example, human NoV RNA could be detected from environmental samples taken during a prolonged outbreak (occurring over the course of ~6 months) in a British hotel (19). Indeed, it is now believed that virus deposited on furniture and carpets, particularly in association with vomiting incidents, is an important vehicle for outbreak propagation (68). In a recent survey, finger nails and clothing were found to facilitate the spread of

virus (6, 82, 107). Their stability on the human skin (2 hours on finger pads with no significant loss in RT-qPCR signal) has also been documented (69).

Lab-based studies using cultivable surrogates have produced mixed results with respect to the environmental persistence of infectious virus. These studies are summarized in Table 1.1, and are difficult to compare due to lack of consistency in experimental design, including the use of different surrogates (FCV, MNV-1), matrices (feces, food, cell culture lysate), and exposures (temperature, pH, moisture, light). Several common themes do emerge, however. For example, both FCV and MNV-1 demonstrate enhanced environmental persistence when suspended in feces as compared to unsupplemented cell culture lysates (14); they are also more stable under moist vs. dry conditions (14). In general, FCV is more sensitive to extremes of pH, while MNV-1 is more sensitive to dry conditions (14). On surfaces, the surrogates can persist for weeks (62), while on foods, they persist for 7-10 days or more (14, 63, 77). Interestingly, FCV was rapidly depurated from oyster samples while human NoV were able to persist for more than 10 days (62).

1.5. RESISTENCE TO PHYSICAL INACTIVATION METHODS

Table 1.2 and 1.3 summarizes the data on the susceptibility/resistance of human NoV and their surrogates to physical inactivation methods commonly used in food preservation and processing. In general, human NoV are quite resistant to many commonly used food processing techniques, including heat, reduced pH, and high

pressure. A high degree of acid resistance would be anticipated, since human NoV must survive the low pH of the stomach in order to reach target cells in the small intestine. Epidemiological evidence supports their acid resistance, as evidenced by the implication of human NoV in a gastroenteritis outbreak associated with contaminated orange juice (pH 3.6) (108).

Heat is perhaps the most commonly used food processing and preparation technique, and the mechanism of virus inactivation by heat appears to be denaturation of the virus capsid, resulting in release of viral RNA (4, 31, 104). Baert et al. (2008) demonstrated a 6.5 $\log_{10}$ reduction in MNV-1 infectivity after exposure of cell culture lysate to 80°C for 150 sec; in this case, RT-qPCR was unable to distinguish between infectious and noninfectious virus after delivery of heat. Similar findings were reported when heat was applied to FCV- contaminated shellfish, in which case a 2 $\log_{10}$ inactivation was observed after immersion of the product in boiling water for at least one minute. Again, RT-qPCR was unable to discriminate between infectious and non-infectious virus (103). An alternative to RT-PCR, nucleic acid sequence based amplification (NASBA) was used to evaluate the survival of human NoV and FCV on artificially contaminated food surfaces (turkey and lettuce) subsequently exposed to 72°C. By infectivity assay, FCV titers dropped by ~6.5 $\log_{10}$ within four minutes of heat treatment while human NoV RNA was still detected by NASBA after a similar treatment (62).

Ultraviolet radiation, widely used in drinking water treatment, has been shown to target the viral nucleic acid by inducing the formation of pyrimidine dimers and other photoproducts, producing nucleic acid lesions that inhibit transcription and replication (43, 44). Studies have clearly demonstrated the efficacy of UV radiation against human NoV. For example, Duizer et al. (2004) demonstrated that the cultivable surrogate canine calicivirus and FCV in cell culture lysate were inactivated 2 $\log_{10}$ when treated with 21 and 22 mJ/cm^2, respectively; a 3 $\log_{10}$ inactivation was observed for both viruses at 34 mJ/cm^2; Human NoV were slightly more resistant than the surrogates, as evaluated by RT-PCR (31). Note that, comparatively speaking, $<$20 mJ/cm^2 is required for a 3 $\log_{10}$ inactivation of bacteria and (oo)cysts of *Cryptosporidium* and *Giardia* in water (44). Lee et al (2008) observed that MNV-1 infectivity was reduced by ~3 $\log_{10}$ after exposure to UV light at 254 nm and 25 mJ/cm^2 [maximum UV absorbance of DNA is at 260 nm (44)], but parallel RT-qPCR assays suggested only minimal inactivation, something of a conundrum given the proposed mechanism of action of UV (63).

Ozone gas, delivered as an aerosol, has been shown to be highly effective against human NoV in surface disinfection of hard (e.g., plastic, steel, glass) and soft (e.g., fabric, cotton, carpet) surfaces (45). However, its high toxicity, poor stability, and explosive nature limit its practical use (53).

Ionizing radiation is a very effective method for elimination of important Gram negative bacterial pathogens from all sorts of foods. It is generally accepted, however,

that the method is relatively ineffective against viruses, at least at practical application doses. For example, Mallet et al. (1991) demonstrated that a 3.0 kGy treatment produced a 95% inactivation of HAV in molluscan shellfish, but unfortunately, this also resulted in significant degradation of the sensory quality of the product (75). Bidawid et al. (2000) reported that a 2.7-3.0 kGy treatment would be needed to achieve only a one $\log_{10}$ reduction of HAV in fruits and vegetables(10).

The application of high pressure for post-harvest elimination of pathogenic *Vibrio* species in molluscan shellfish has attracted recent interest. This method has the advantage of achieving pathogen inactivation while allowing retention of product sensory quality. In general, enveloped viruses are more sensitive to elevated pressure, displaying complete inactivation at pressures >450 MPa (10). Non-enveloped viruses such as human NoV would be expected to be more resistant. Chen et al. (2005) studied FCV inactivation kinetics upon exposure to high pressure, finding that degree of inactivation depended on three factors: pressure, temperature and time. This lead to the hypothesis that virus inactivation by high pressure occurred as a result of protein denaturation and hence capsid disruption (20). Similar results were reported by Buckow et al. (2008) who found that a 250 MPa treatment resulted in a 5 $\log_{10}$ reduction of FCV cell culture lysate (12). Kingsley et al. (2002) investigated the impact of high pressure on HAV and FCV, finding that pressures commonly used for *Vibrio* control (200-250 MPa) were unable to completely inactivate these viruses(57). However, pressures ≥450 MPa resulted in inactivation exceeding 7 $\log_{10}$ PFU/ml. Later work by the same

investigators demonstrated a 4 log_{10} inactivation of MNV-1 after a 5 minute exposure to 400 MPa at 5°C (56).

1.6. RESISTANCE TO CHEMICAL DISINFECTANTS

The U.S. Centers for Disease Control and Prevention defines a disinfectant as a chemical agent applied to inanimate objects that destroys harmful microorganisms and pathogens, but does not destroy spores (17). The difference between a disinfectant and an antiseptic is that the latter is for use on living tissues, and can have either microbiostatic or microbiocidal activity (17). On the other hand, a sanitizer is an agent that reduces the bacterial load to “safe” levels. In the U.S., both the EPA and FDA regulate liquid chemical disinfectants; EPA regulates disinfectants used on environmental surfaces, while FDA regulates those used on medical devices (16). The EPA specifies a sanitizer as a chemical able to eliminate 5 log_{10} (99.999%) of a specific test bacterium in 30 seconds under normal use conditions (17). In order for a disinfectant to claim antiviral efficacy, it must demonstrate complete inactivation of at least a 10^4 recoverable virus titer or a 3 log_{10} reduction beyond cytotoxicity levels (17). At the time of this writing, FCV is specified by EPA for use as the surrogate for human NoV (17).

1.6.1. Inactivation of human NoV in Water

Chlorine compounds have a long history of use in water disinfection, particularly sodium hypochlorite (note: sodium hypochlorite is also referred to as free chlorine, due to its tendency to dissociate from sodium and oxygen, producing free chlorine ion) and monochloramine. Sodium hypochlorite is the active compound in bleach and behaves as a very strong oxidizing agent, with its degree of activity dependent upon both concentration and contact time (6). The antiviral activity of free chlorine is believed to occur at both the capsid and RNA levels (79). For example, O'Brien and Newman (1979) reported that free chlorine inactivates poliovirus by cleaving RNA while it was still associated with the viral capsid (87). Li et al. (2002) suggested that free chlorine affected the viral nucleic acid because the infectivity of HAV was completely lost in a concentration and time dependent manner, but antigens remained intact (64).

There have been many, many studies designed to evaluate the efficacy of chlorinated water against various human enteric viruses. The interested reader is referred to pertinent reviews by Deborde and Gunten (2008) and Clasen et al. (2007) (24, 28). Here, we briefly discuss studies specifically focused on inactivation of NoV in water. Both commonly used human NoV surrogates appear to be rapidly inactivated in chlorinated water (25). Interesting, Keswick et al. (1985) suggested that the resistance of human NoV to chlorine in drinking water, as observed by human challenge study, occurred in part because of virus aggregation (52). This hypothesis was recently

supported by findings that purified, dispersed human NoV is more sensitive to free chlorine than aggregated human NoV (109).

Monochloramine is produced when chlorine is added to a solution containing ammonia; this occurs spontaneously as a consequence of drinking water chlorination or alternatively, monochloramine can be added to water. Monochloramines generally have less antiviral activity than free chlorine, and human NoV are more resistant to monochloramine than are adenoviruses and enteroviruses (25). However, monochloramines continue to be used because they are less inclined to result in the production of toxic trihalomethanes (112); monochloramines are most often used as a secondary disinfectant.

Chlorine dioxide is also used for water disinfection. In early studies, it was suggested that its mechanism of antiviral activity was disruption of capsid protein, perhaps resulting in alteration of the ability of the virus to attach to host cells (112). However, others have suggested that chlorine dioxide damages viral RNA. For example, exposure to chlorine dioxide impaired the replication process of poliovirus and did not appear to cause structural damage to the virus capsid as evaluated by electron microscopy (1). A more recent study employed infectivity assay, enzyme linked immunosorbent assay (ELISA) and RT-PCR to study the infectivity, antigenicity and genome integrity of HAV after exposure to chlorine dioxide. Results showed that a treatment with 7.5 ppm chlorine dioxide for 10 minutes resulted in complete loss of HAV infectivity. Based on these results of this study, the mechanism of inactivation

was assumed to be at the capsid level because antigenicity was lost (as determined using ELISA). However, the viral nucleic acid was also damaged as evidenced by a failure to detect viral RNA using RT-PCR (65). Recent work by Simonet and Gantzer (2006) supports this finding(101). These investigators compared the effects of chlorine dioxide on intact poliovirus and extracted viral RNA, finding a dose-dependent relationship for degradation of both capsid and nucleic acid.

Ozone is widely used in Europe for drinking water disinfection. As a strong oxidant, it was initially believed that ozone's mechanism of inactivation involved oxidative degradation of viral capsid proteins (53). This is supported by recent work of Lim et al. (2010), who monitored the efficacy of ozone for inactivation of MNV-1, as evaluated by both RT-PCR and infectivity assay(66). These investigators observed that RT-PCR significantly underestimated the degree and rate of MNV-1 inactivation. However, others have suggested that ozone actually targets the viral RNA (93), as evidenced by the fact that survival of poliovirus and bacteriophage MS-2 exposed to various ozone concentrations was similar when evaluated by both infectivity assay and RT-PCR (100). It may be that ozone is so effective because it targets both viral RNA and capsid protein (55).

Recently, there has been quite a bit of interest in the use of biogenic silver-based nanoparticles for water disinfection. Biogenic silver is produced using the natural capability of lactic acid bacteria to reduce ionic silver into zerovalent (biogenic) silver, with the size of the resulting silver nanoparticle dependent upon the location in the

bacterial cell from which the nanoparticle was recovered (e.g., adjacent to the cell wall vs. cytoplasmic location), as well as the species of lactic acid bacteria used (27, 102). In a recent study by De Gusseme et al. (2010), *Lactobacillus fermentum* was used to both reduce and serve as a matrix to carry zerovalent silver which was immobilized on an electropositive filter and used for continuous water disinfection. When challenged with MNV-1 contaminated water, the investigators observed a 3.8 $\log_{10}$ reduction to virus titer, which under the conditions of the study, constituted complete virus inactivation. Overall, they observed that smaller particles performed better than larger ones. The suggested mechanism of activity of biogenic silver-based nanoparticles involves capsid protein disruption by silver, which results in exposure of viral RNA to environmentally ubiquitous RNases (27).

1.6.2. Inactivation of human NoV on Surfaces (including foods)

Historically, chlorine-based products have been important tools for the food processing and retail industries, particularly for surface disinfection. More recently, hypochlorite has also been used for decontamination (washing) of foods such as freshly slaughtered meat carcasses and fresh or fresh-cut produce. For these sorts of applications, the most common forms of chlorine are hypochlorous acid and its salt derivative, sodium hypochlorite. The hypochlorite concentration in undiluted household bleach is 50,000 ppm chlorine; it is generally recognized that when diluted to concentrations $\geq$5,000 ppm, it will be effective in inactivation of human NoV (6).

However, federal regulations prohibit the food industry from using solutions exceeding 200 ppm for sanitation of food processing equipment or food contact surfaces (21 CFR Part 178). Higher concentrations may be used, but the surface must be rinsed with potable water and higher concentrations may also cause corrosion of equipment.

A number of studies have sought to characterize the efficacy of hypochlorite in inactivating human NoV and their surrogates from contaminated surfaces. As a general rule of thumb, relatively high concentrations of sodium hypochlorite are needed to significantly reduce the titer of human NoV and the surrogates when dried onto surfaces. For example, when FCV suspended in 5% organic material (simulated using FBS) was dried onto sterile plastic petri dish surfaces that were then treated for 10 minutes as 100 and 1,000 ppm, Jimenez and Chiang (2006) observed 3.2 and 6.6 $\log_{10}$ reductions, respectively, as determined by infectivity assay (48). Gulati et al. (2001) observed minimal (1 $\log_{10}$) reduction of FCV (by infectivity assay) after treatment of contaminated stainless steel disks with <800 ppm free chlorine, whereas treatment with 5,000 ppm resulted in a 3.4 $\log_{10}$ reduction after contact times of 1 to 10 minutes (41). Whitehead and McCue (2010) found that treatment of dried FCV with 1,000 ppm free chlorine for 1 minute resulted in >3 $\log_{10}$ inactivation(111). Barker et al. (2004) reported that, after cleaning contaminated melamine surfaces with a detergent, a one minute exposure to 5,000 ppm free chlorine significantly reduced the likelihood of residual Norwalk virus contamination. However, previous mechanical action to reduce soil load was critical to effective disinfection. Based on data such as these, the CDC

recommends a minimum of 1,000 ppm for disinfection of surfaces contaminated with human NoV (CDC, 2006).

Hypochlorous acid has been tested as a liquid for surface disinfection, and also as a fog. In suspension and surface (ceramic and stainless steel) disinfection studies employing human NoV and two surrogates (MNV-1 and bacteriophage MS2), application of 20-200 ppm hypochlorous acid resulted in $\geq 3 \log_{10}$ reduction of all viruses after exposure for 10 minutes, as evaluated by infectivity assay and RT-PCR, as appropriate. Similar results were observed when hypocholorous acid was used as a fog and applied in a confined space (88).

Chlorine compounds are readily inactivated by organic matter, so the fact that human NoV are fecally associated may actually protect the viruses from inactivation by hypochlorite. Human NoV also tend to attach to organic matter, a phenomenon that may shield virus from contact with the disinfectant (109). For example, Urakami et al (2007) observed a $>4 \log_{10}$ reduction in the infectivity of partially purified FCV after a five minute exposure to a concentration of 300 ppb (or 3.0×10^{-3} ppm) free chlorine. However, the efficacy of inactivation changed as a function of protein concentration (as measured by residual host cell debris) such that solutions with higher protein content showed less relatively poorer disinfection efficacy (109). Curiously, in comparative studies in which human NoV surrogates were suspended in relatively clean (cell culture lysates) and dirty (artificial feces or FBS) matrices, hypochlorite inactivation rates were

not significantly different by matrix (18). It has been proposed that these artificial matrices may not be entirely representative of the complexity of natural feces (109).

Given the above issues with organic matter, it is no wonder that chlorine compounds perform poorly when used for inactivation of viruses contaminating the surface of fresh produce. For example, a 2 $\log_{10}$ reduction in MNV-1 was observed after washing iceberg lettuce in water supplemented with 200 ppm sodium hypochlorite (Baert et al. (2009)), and sodium hypochlorite concentrations as high as 800 ppm were no better than water rinses for inactivation/removal of FCV inoculated onto the surfaces of strawberries and lettuce leaves (3, 41).

Quaternary ammonium compounds (QUATS) are another commonly used commercial disinfectant category. The EPA stipulates that ≤ 400 ppm of QUAT residue may be present on end-use product (food) (40 CFR Part 180), but 200 ppm is more commonly used since it is the lowest recommended use concentration without requiring a water rinse. Many studies have reported the relative lack of efficacy of QUATS against non-enveloped RNA viruses, which is intuitive as they are lipophilic compounds and the human enteric viruses lack a lipid envelope (39). There is some evidence that the addition of sodium bicarbonate to QUAT formulations provides improved antiviral efficacy against FCV, but this finding may be more a function of the elevated pH of the resulting formulation rather than efficacy of the active compound (30, 73).

1.6.3. Inactivation of human NoV on Hands

Given the significant epidemiological association between human NoV outbreaks and foods receiving a high degree of human handling prior to consumption (7), human hands remain important contamination routes. The active agent in most of the commercially available hand sanitizers is ethanol or isopropanol used at concentrations of 60-95% (34). The antimicrobial efficacy of alcohols is based on their lipophilic nature (8), so theoretically, alcohols would not be expected to be very effective against non-enveloped viruses.

However, there is conflicting evidence in this regard, particularly in experiments that have used human NoV surrogates. For example, Belliot et al. (2008) observed a 4 $\log_{10}$ reduction in MNV-1 when exposed to two different ethanol-based hand sanitizers(8). Likewise, Gehrke et al. (2004) reported similar efficacy of 1-propanol against FCV, with a median concentration of 70% producing the best results(37). On the other hand, Macinga et al. (2008) reported $<1 \log_{10}$ reduction of MNV-1 when exposed to 75% ethanol, while Lages et al. (2008) found minimal efficacy of ethanol (62% in hand gel and 99.5% ethanol) against FCV ($\leq 1 \log_{10}$ reduction after a 30 second contact time) (60, 72). It has been suggested that high alcohol concentrations may be less effective than lower concentrations because of rapid evaporation which results in reduced contact time (37, 60, 74). When alcohols do work, it is believed that their mechanism of inactivation is at the level of the viral capsid because ethanol has been hypothesized to denature proteins (79).

Triclosan is a polychloro-phenoxy phenol compound that is a common component of many antimicrobial hand products; it has been suggested that Triclosan exerts its antimicrobial efficacy at the level of the bacterial cytoplasmic membrane; it may also block fatty acid synthesis (70, 79). Like alcohol, Triclosan-based hand soaps would be expected to have poor efficacy against non-enveloped enteric viruses and indeed they produce low reductions of Norwalk virus, comparable to what is observed for a simple tap water rinse (70). The previous authors and others have suggested that reduction in human NoV contamination on hands treated with traditional hand sanitizers is due more to the mechanical removal of virus rather than to any specific antiviral activity of the “active” compound(s) (60, 78, 94).

1.6.4 Direct Comparison of human NoV and its Surrogates

Most studies undertaken to evaluate the efficacy of human NoV inactivation strategies have been applied either solely to human fecal suspensions using RT-qPCR, or else to surrogates whose survival was assessed exclusively by infectivity assay. Very few studies have sought to combine RT-qPCR with infectivity assay (for surrogates), or to compare surrogates with human NoV strains. These sorts of experimental designs, however, would provide the most valuable information because they entail direct comparison of human strains to surrogate strains, and/or of RT-qPCR to infectivity assay. A few relevant (recent) studies are described below.

Using highly purified MNV-1, human NoV strain GII.4, and poliovirus, Kitajima et al. (2010) evaluated the antiviral efficacy of sodium hypochlorite used in water disinfection(58). By RT-qPCR, the investigators observed a 3.6 $\log_{10}$ inactivation of human NoV GII.4 and a 2.9 $\log_{10}$ reduction of MNV-1 after exposure to 0.5 ppm free chlorine for a maximum contact time of 30 minutes. By infectivity assay, MNV-1 titer was reduced >4.4 $\log_{10}$ under the same conditions (39, 58). Girard et al (2010) compared the efficacy of surface disinfection (stainless steel) as applied to MNV-1 and human NoV, finding that 30,000 ppm sodium hypochlorite was most effective than ethoxylated alcohol or quaternary ammonium, resulting in 4 $\log_{10}$ reduction of MNV-1 (by plaque assay and RT-qPCR), and a 3 $\log_{10}$ reduction for human NoV (by RT-qPCR) (39).

1.7. DISCRIMINATION OF INFECTIOUS VS. NONINFECTIOUS VIRUS

The relative inability of RT-qPCR to discriminate between infectious and non-infectious virus hinders the ability to reliably predict the environmental behavior and inactivation efficacy of candidate control measures targeting human NoV. Over the years, various investigators have sought ways to harness RT-qPCR and at the same time, confirm virus infectivity. These have constituted two general approaches, i.e., (i) those estimating capsid integrity; and (ii) those evaluating genome integrity. These are discussed briefly below.

1.7.1 Methods Targeting Capsid Integrity

Methods that combine *in vitro* amplification of viruses in tissue culture with subsequent molecular amplification (integrated cell culture-RT-PCR) are well suited for viruses that will replicate but may not produce visible cytopathic effects. An additional advantage of this method is that it proves the presence of infectious virus, albeit only qualitatively. This approach has been used widely for detection of HAV in contaminated environmental and food samples (water and oysters) (22, 47). In one study, investigators used integrated cell culture-RT-PCR to evaluate the efficacy of chlorine against poliovirus, finding a reduction in false negative results when compared to conventional cell culture assay (11). Integrated cell culture-RT-PCR is, of course, not relevant for human NoV, although it is a useful screening tool in continuing efforts to develop cell culture models for these viruses (11). Major drawbacks to integrated cell culture-RT-PCR is that it is expensive, time consuming and labor intensive, certainly not amenable to routine diagnostic applications.

A method that has gained considerable popularity is sample pre-treatment with proteinase K and/or RNase just prior to RT-PCR. This approach targets both damaged viral capsid, which is more susceptible to proteinase K digestion, and free or otherwise exposed RNA, which will be digested by RNase. Nuanualsuwan and Cliver (2002) were the first to use this method, demonstrating a negative RT-PCR result after using heat, UV, and hypochlorite to inactivate HAV, poliovirus, and FCV(86). The same investigators used the method to demonstrate that HAV and FCV lost their ability to

attach to host cell receptors after treatment with UV, hypochlorite, and heat, suggesting that capsid-associated changes occurred as a result of exposure to these treatments (85). This method has been recently combined with NASBA in studies evaluating the persistence of human NoV on stainless steel and polyvinyl chloride (PVC) surfaces. In this case, pretreatments were reported to be effective because viral RNA copy number (and hence "potentially" infectious NoV particles) decreased over time, a trend similar to that observed in infectivity assays applied to surrogate viruses (61). Also, in an earlier study, Lamhoujeb et al. (2008) found that when preceding NASBA with enzymatic treatments, heat inactivated human NoV failed to be detected (62).

However, other studies report conflicting results, finding enzymatic pretreatment is not necessarily a reliable means by which to predict virus infectivity. For example, Baert et al. (2008) observed that high titers of MNV-1 RNA could still be detected by RT-qPCR after heat treatment even though no infectious virus could be quantified (4). Similarly, Poschetto et al. (2007) noted that RNase and proteinase K pretreatments did not reduce the RNA concentration for FCV exposed to sodium hypochlorite even though infectivity assay demonstrated virus inactivation (92). Liu et al. (2010) also found no statistically significant differences between genomic copies of Norwalk virus detected with or without enzymatic pretreatments after exposure to alcohol-based hand sanitizers (70).

Antibody-bound magnetic beads have been widely used to aid in the concentration of enteric viruses from complex matrices such as food, environmental, or

clinical (fecal) samples in preparation for detection by RT-PCR (49, 59, 89, 97). For example, Park et al. (2008) used immunomagnetic separation (IMS) combined with RT-qPCR to recover and detect human NoV from artificially contaminated strawberries, achieving recovery rates ranging from 14-30% (89). Relatively low recovery rates (<10%) were observed by Kim et al. (2008) in their attempts to detect human NoV from grapes, strawberries and raspberries(54). It has been argued that, in addition to facilitating virus concentration with removal of matrix-associated inhibitors, the use of IMS may correlate with recovery of infectious virus (38). Although not a direct measure of virus infectivity, antibody capture does circumvent the problem of detection of naked viral RNA, which can result from capsid disruption occurring as a consequence of virus inactivation (38, 49).

One of the problems often reported for IMS is a tendency toward non-specific binding of antibody-bound paramagnetic beads to residual matrix-associated components as Gilpatrick et al (2000) found that HAV was detected along with human NoV (38). However, this did not have an affect on the detection limit of the assay. Another problem unique to human NoV is their broad antigenicity, meaning that antibodies raised against one strain are frequently ineffective for the capture of others. Further, only a small number of human NoV antibodies are commercially available. Although Gilpatrick et al. (2000) successfully demonstrated that human hyperimmune serum could be used for the capture of a broader range of human NoV, this method still

lacks broad reactivity (38). It is further limited by relatively short-lived immunity to human NoV and the high expense of pooled human immunoglobulins (38).

Recently, other ligands have been considered as alternatives to antibodies. For instance, based on recent evidence that human NoV have a high degree of binding affinity to glycopeptides moieties associated with the human histo-blood group antigens (HBGAs) synthetic HBGAs have been used as capture ligands (42). Biotinylated HBGAs are commercially available, and these have been attached to streptavidin-coated magnetic beads to facilitate capture of human NoV from environmental water (15). More recently, Morton et al. (2009) were able to concentrate multiple human NoV strains from food samples using this technique (83). Other potential ligands might include peptides and nucleic acid aptamers, the latter of which are single stranded oligonucleotides that have target-specific binding affinity when folded into their native three-dimensional structures (5). Theoretically, alternative ligands such as these would have binding affinity and specificity properties similar to those of antibodies, but with the added advantage of ease of production, better stability, and broader reactivity.

1.7.2 Methods Targeting Genome Integrity

DNA intercalating agents such as ethidium monoazide (EMA) and propidium monoazide (PMA) have been used in conjunction with qPCR to discriminate live bacterial cells from dead ones (84). These compounds selectively penetrate the membranes of dead bacterial cells and form stable DNA monoadducts upon photolysis,

resulting in DNA structures that cannot be amplified by PCR. Investigators have recently reported the use of PMA to distinguish between infectious and non-infectious viruses (Norwalk virus, poliovirus, coxsackievirus and echovirus) that were inactivated by exposure to sodium hypochlorite or heat (90). In this case, PMA was used as a pre-treatment prior to RT-PCR, with a presumed mechanism of action similar to that for bacterial cells. While effective for discriminating poliovirus infectivity status, the method did not appear to work well for human NoV as high virus titers were observed by RT-qPCR both with and without PMA pre-treatment, even after the application of extreme conditions that would result in virus inactivation.

1.8 CONCLUSIONS

Human NoV are the most common cause of acute viral gastroenteritis worldwide (40), and they continue to be the leading cause of food borne disease (96). A number of factors contribute to the ease with which these viruses are transmitted, including multiple transmission routes, low infectious doses, incomplete immunity, and high virus loads shed during infection. There remain many challenges in our efforts to study and control human NoV. The tendency of RT-pPCR assays to underestimate the efficacy of candidate control strategies, largely because of the poor correlation between detection of the viral genome and corresponding infectivity, has complicated our efforts in this regard. In the absence of cultivable human strains, surrogate viruses have been

widely used, but increasing evidence reveals that their behavior may not always be representative of that of human NoV.

Despite these difficulties, several pieces of evidence are undisputed. Firstly, human NoV are resistant to many commonly used physical inactivation methods (e.g., heat, UV light, ionizing radiation, high pressure), at least as evaluated by RT-qPCR. Likewise, chemical disinfectants have minimal efficacy at commonly used concentrations. Only sodium hypochlorite (at high concentrations >1,000 ppm) and ozone show substantial efficacy, but both these treatments have significant disadvantages. Hand sanitizers containing ethanol and Triclosan are relatively ineffective against human NoV.

Together, the methodological complications and the poor efficacy of existing inactivation and disinfection methods have important implications for controlling the transmission of human NoV through the farm-to-fork continuum. There is a clear need for better microbiological methods: a cultivable human NoV strain and in the absence of this, more relevant surrogates, as well as molecular-based methods that also reliably discriminate between infectious and non-infectious viruses. These methodological developments are essential as we seek to identify novel disinfectants with improved anti-noroviral efficacy. Only with more efficacious disinfectants, along with better user compliance, will we be able to really tackle the problem of food borne human NoV transmission, particularly in association with infected food handlers.

Table 1.1 Persistence Studies

Virus	Treatment	Assay	Result	Reference
MNV-1	virus survival on diaper, gauze and in human stool up to 40 days at various temperature conditions (-20, 4, 18, 30'C)	infectivity assay and RT-qPCR	MNV-1 survived >40 days at low temperatures and best in stool suspension	Lee et al.(2008)
FCV, HuNoV	persistence on refrigerated RTE food	NASBA; infectivity assay	NoV survived at least 10 days on RTE food; FCV more sensitive to heat and enzymatic pretreatment than HuNoV	Lamhoujeb et al.(2008)
HuNoV GI and GII	finger pad, ceramic, formica, stainless steel	RT-qPCR	RNA detected by RT-PCR 28 days after innoculation of surfaces	Liu et al.(2009)
MNV-1, FCV	virus stocks reconstituted in artificial feces and dried on stainless steel coupons up to a week	infectivity assay	MNV-1 and FCV inactivated at similar rates except under wet conditions, MNV-1 was more stable	Cannon et al.(2006)
HuNoV, FCV	persistence on stainless steel, formica, ceramic coupons and transfer to lettuce at different pressure and time	infectivity assay and RT-qPCR	HuNoV and FCV detected on all 3 surfaces after 7 days. FCV had a 6-7 log drop in titer after 7 days; purified HuNoV RNA did not persist after 24 hours	Mattison et al. (2007)
MNV-1, FCV, HuNoV GI	persistence in surface and ground water at 4'C and 25'C over 3-5 weeks	infectivity assay and RT-qPCR	MNV-1, MS2 and HuNoV GI nucleic acid showed 0.04 log reduction/day; FCV had higher reduction than all other viruses; infectivity reduction was higher than nucleic acid reduction at 25'C	Bae and Schwab (2008)
FCV in human fecal suspension	persistence on lettuce, strawberries, ham, metal disk	infectivity assay	virus survived better at 4'C than room temperature; ham was best for FCV survival	D'Souza et al. (2006)
FCV, purified HuNoV GII	RNA persistence in oysters	RT-qPCR	HuNoV RNA was still detected after 10 days while FCV was rapidly depurated	Ueki et al. (2007)
FCV	persistence of FCV in a natural fecal suspension on surfaces (stainless steel) and food (lettuce, strawberries, ham) over 7 days at room and refrigeration temperatures	infectivity assay	At room temperature, FCV was recovered at for ham and stainless steel for all days, FCV undetectable for lettuce and strawberries after 4 and 2 days respectively; at refrigeration temperatures, FCV was recovered on all surfaces for all seven days except for strawberry (undetectable after 6 days)	Mattison et al. (2007)

Table 1.2 High Pressure Processing Studies

Virus	Treatment	Assay	Result	Reference
FCV	high hydrostatic pressure (200 and 250 Mpa) and temperature	immunochemiluminescent focus assay, infectivity assay	pressure treatment most effective at temperatures above and below 20'C, 200 Mpa showed more tailing of inactivation curve	Chen et al.(2005)
FCV	FCV inactivation in suspension (cell culture medium and mineral water); ambient pressure at 75'C for 2 minutes and 450 Mpa at 15'C for 1 minute	infectivity assay	FCV inactivated >7 log for both treatments	Buckow et al. (2008)
FCV, HAV, poliovirus	high pressure treatment (5 or 15 minutes with pressures ranging between 300 and 480 MpA)	RT-PCR and infectivity assay (HAV)	FCV inactivated after 5 minutes of 275 Mpa to nondetectable levels;	Kingsley et al. (2002)
MNV-1	oysters, high pressure processing (350 and 450 Mpa)	Infectivity Assay	MNV-1 in suspension was inactivated 6.85 log after 5 minutes of 450-Mpa at room temperature; 1.15 log after 5 minutes 350-Mpa at 30'C; and 5.56 log after 5 min 350-Mpa at 5'C. In oyster tissue, 400 Mpa after 5 minutes inactivated MNV-1 4.05 logs	Kingsley et al. (2007)
MNV-1	400 Mpa for 5 minutes use of proteinase K and Rnase A	infectivity assay and RT-qPCR	MNV-1 declined 2 log after 400 Mpa for 5 minutes, HPP did not damage MNV-1 RNA	Tang et al. (2010)
MNV-1, HuNoV GII.4	450 Mpa for 15 minutes at 45'C	infectivity assay (TCID50), RT-qPCR	6.5 log reduction in infectious MNV-1, RNA titer decreased proportionally to infectivity but at a lower rate	Sanchez et al. (2011)
MNV-1	oysters contaminated with MNV-1, treated with 5 minutes 400 Mpa	in vivo study; fed to mice	HPP treated MNV-1 did not cause infection	Gogal et al. (2011)

Table 1.3 Heat Treatment Studies

Virus	Treatment	Assay	Result	Reference
CaCV, FCV, HuNoV GII.4	suspension assays,exposure to 4, 20, 27, 37, 56, 63, 71.3, 100'C ranging from seconds to weeks	RT-qPCR and TCID50	4'C inactivated CaCV and FCV <1 log after 2 weeks; 20'C after 1 week achieved 3 log reduction; 3 log reduction observed for 37'C at 24 hours and 56'C for 8 minutes; by RT-qPCR showed >5 log for CaCV and FCV after exposure to 37'C up to 168 hours, HuNoV GII.4 showed <1 log reduction, 100'C for 3 minutes showed considerable RNA reduction for all three viruses	Duizer et al. (2004)
MNV-1	heat exposure of 80'C and Rnase and proteinase K were used over 6 minutes	RT-qPCR and infectivity assay	80'C at 150 seconds resulted in 6 log reduction by infectivity assay but by RT-qPCR, 9 logs of genomic copies were detected; reduction of infectious particles does not correlate to number of genomes detected by RT-qPCR; heat affected infectivity more than viral genome integrity	Baert et al. (2008)
MNV-1, HuNoV GI and GII	63' and 72'C at 1,2,5 and 10 minutes in water and milk	RT-qPCR and infectivity assay	>2 minutes in water at 63'C showed >3 log reduction for MNV-1 infectivity, all contact times showed >3.5 log reduction at 72'C in water for MNV-1 infectivity; by RT-qPCR, MNV-1, HuNoV GI and GII showed <1 log reduction for both temperatures at all times	Hewitt et al. (2009)
HAV, MNV-1	90, 180, 300 seconds, 85-90 'C	infectivity assay and RT-qPCR	By RT-qPCR, 90'C for 90 seconds reduced MNV-1 by 3.3 logs; by infectivity assay 85'C for 180 seconds reduced 5.47 logs	Sow et al. (2011)
FCV	heat treatment in cockles, boiling for 30 seconds, 1 min, 1.5 min, 2 min, 2.5 min and 3 min	RT-PCR, TCID50	2 log reduction in infectivity after 30 seconds; no virus was recovered after 1 minute of heat treatment; no FCV amplimer detected after exposure to >2 minutes	Slomka and Appleton (1998)
FCV	56'C, 70'C or boiled for 1, 3, 5, 60 minutes	TCID50	56'C at 60 minutes showed 7.5 log reduction; 70'C showed 3, 6.5 and 7.5 log reduction at 1, 3, 5 minutes respectively; boiling for 1 minute showed 7.5 log reduction	Doultree et al. (1999)

Table 1.4 Ethanol Disinfection Studies

Virus	Treatment	Assay	Result	Reference
MNV-1	suspension test with ethanol and isopropanol 10, 30 or 60%)	infectivity assay, RT-qPCR	60% ethanol and isopropanol showed 4 and 3.5 log reduction after 30 seconds, 1 minute and 3 minute contact times	Belliot et al., 2008
FCV	50, 70 and 80% ethanol at 30s, 1, 3, 5 minutes in suspension; 10, 20, 30, 40, 50, 60, 70, and 80% at 30s and 1 minute; finger pad method with 70 and 90% ethanol for 30 seconds	TCID50	50 and 70% produced log reduction of 4.13 and 4.06; extrapolated data showed 1 minute with 67% ethanol would result in 3.8 log reduction; finger pad results showed 70% had 3.8 log reduction and 90% showed 2.8 reduction	Gehrke et al., 2004
FCV	99.5% ethanol and other various hand sanitizers testing on human hands using finger pad method	TCID50	99.5% ethanol showed a log reduction after 1 minute and 1.3 log reduction after 2 minutes	Lages et al., 2008
FCV, MNV-1, HuNoV GII.4	suspension test with 50, 70 and 90% ethanol, contact times of 1 and 5 minutes	infectivity assay and RT-qPCR	infectivity assay: 70% and 90% ethanol reduced MNV-1 by ≥ 3.6 log after 5 minutes; 50 and 70% reduced FCV by > 2.2 log after 5 minutes; RT-qPCR showed >3 log reduction of MNV-1 RNA with 70 and 90% ethanol at 1 and 5 minute exposures; ≤ 1 reduction in RNA for MNV-1 at 50% ethanol and all concentrations for FCV and HuNoV GII.4	Park et al., 2010
MNV-1	suspension test and in vivo test (finger pad)	infectivity assay, RT-qPCR	75% ethanol showed 0.91 log reduction in MNV-1 when tested on finger pads	Macinga et al., 2008
CaCV, FCV	suspension test, 70% ethanol	TCID50	<8min resulted in <2 log reduction for both CaCV and FCV; after 30 minutes, 3 log reduction was observed	Duizer et al., 2004

Table 1.4 cont.)

Virus	Treatment	Assay	Results	References
HuNoV	suspension test and fingerpad; 3, 17, 31, 47, 62 and 95% ethanol	RT-qPCR	<0.5 log reduction for all concentrations	Liu et al., 2010
MNV-1	surface test (stainless steel disc) with 40, 45, 50, 55, 60% ethanol and also under soil conditions (BSA) with contact time of 5 minutes	infectivity assay	concentration effect for 40-60% ethanol (40% showed <1 log reduction, 45% showed 2 log reduction, 50% showed 4 log reduction and >55% showed 6 log reduction), addition of BSA had no effect	Magulski et al., 2009
FCV	surface test (polystyrene Petri dish) with 60% ethanol, contact time of 1 minute	infectivity assay	1.3 log reduction was observed	Whitehead and McCue 2010
FCV	75% ethanol, 1 minute contact time	TCID50	1.25 log reduction was observed	Doultree et al.,1999

Table 1.5 Chlorine Disinfection Studies

Virus	type: surface, food, misc.	Treatment	Assay	Result	Reference
FCV	surface inactivation (polystyrene Petri Dish)	100ppm, 1000ppm chlorine, contact time of 1 minute	infectivity assay	100ppm showed <2.27 log reduction; 1000ppm showed >4.2 log reduction	Whitehead and McCue (2010)
FCV	suspension	10ppm, 0.3ppm free chlorine	TCID50	purified FCV was reduced >4.6 log after 5 minutes to 0.3ppm free chlorine	Urakami et al. (2007)
HuNoV GII	surface (melamine) and finger pad	5000ppm	RT-PCR	residual HuNoV was detected in all melamine surfaces, after cleaning and then exposure to 5000ppm, there was slight reduction in HuNoV positive surfaces	Barker (2004)
FCV	surface inactivation	100ppm and 1000ppm chlorine tested, virus was dried on petri dishes then covered with disinfectant for 10 minutes	infectivity assay	~3 log reduction for 100ppm, ~6.5 log reduction for 1000 ppm chlorine	Jimenez and Chiang (2006)
FCV	surface inactivation	stainless steel, strawberry, lettuce were used as surfaces; 200, 400 and 800 ppm of free chlorine were tested at contact times 1-10 minutes	infectivity assay	contact time of 10 minutes: <800 ppm showed <1 log reduction; 5000ppm showed 3.4 log reduction on food contact surfaces; 800 ppm showed 1.5 log reduction on strawberry and lettuce	Gulati et al. (2001)
FCV	suspension test with 1 minute contact time	100, 250, 500, 1000, and 5000ppm chlorine for 2 different hypochlorite compounds	TCID50	<500ppm showed <3 log reduction; 5000ppm showed 5 log reduction	Doultree et al. (1999)
MNV-1, HuNoV	surface inactivation (stainless steel)	3000 ppm free chlorine	infectivity assay, RT-qPCR	For MNV-1 4 log reduction was achieved by RT-qPCR and complete inactivation by plaque assay; HuNoV: 2 log reduction fter 5 minutes, >3 log reduction after 10 minutes	Girard et al. (2010)

Table 1.5 cont.)

Virus	type: surface, food, misc.	Treatment	Assay	Result	Reference
MNV-1, MS2, HuNoV GII.4	surface inactivation and in suspension	stainless steel and ceramic tiles; HAS as fog and in suspension	infectivity assay, RT-PCR	undiluted and 38 and 18.8ppm reduced RNA titer of HuNoV by 3 log after 20 seconds; surface test fogging showed >3.5 reduction by infectivity assay and >4.5 by RT-PCR for MNV-1; and >4.5 for HuNoV GII.4	Park et al. (2007)
MNV-1	suspension	2600ppm chlorine with contact time 30 seconds and 1 minute	infectivity assay, RT-qPCR	>4 log reduction for both infectivity assay and RT-qPCR	Belliot et al. (2008)
CaCV, FCV, HuNoV GII.4	suspension	30 ppm, 300ppm, and 3000ppm chlorine	TCDI50, RT-qPCR	<30ppm resulted in <1 log reduction; 300ppm resulted in 3 log reduction for CaCV and 2 log reduction for FCV after exposure for 10 minutes; complete inactivation (>5 log) was observed for CaCV and FCV with 3000ppm free chlorine in 10 and 30 minutes at room temperature; by RT-qPCR reduction in RNA was similar to loss of infectivity	Duizer et al. (2004)
HuNoV	suspension	3, 22, 51, 160 and 1600ppm	RT-qPCR	log-linear reduction observed for concentrations 22-160ppm after 30 second exposure; 5 log reduction for 160 and 1600 ppm	Liu et al. (2010)
HuNoV GII, FCV	suspension test with and without soil (FBS or feces) for FCV	4500-5000ppm chlorine and proteinase K and Rnase	TCID50 and RT-qPCR	FCV: without feces >5 log reduction observed for 4500-5000 ppm after 15 minute contact time and 1000 ppm after 2 hours; with feces, 4 log reduction observed for 5500ppm. By RT-PCR, 1000ppm had no effect on FCV RNA, 5500, 6000, 7000 had no effect on FCV or HuNoV with feces/protein load; 5000 ppm yielded >4 log without feces. Overall, reducing feces load made hypochlorite concentrations effective for both FCV and HuNoV (3 log reduction)	Poschetto et al. (2007)

Table 1.6 Quaternary Ammonium Disinfection Studies

Virus	Treatment	Assay	Result	Reference
HuNoV, MNV-1	0.08% N-alkyl dimethyl benzyl ammonium chloride; 0.02% N-alkyl dimethyl benzyl ammonium chloride	infectivity assay and RT-qPCR	MNV-1: 1 log reduction after 10 minutes, and <0.5 log reduction after 5 minutes with both plaque assay and RT-qPCR; no effect on HuNoV	Girard et al. (2010)
FCV	Quaternary ammonium (1:10) Pinocleen)	TCID50	no log reduction observed	Doultree et al. (1999)
FCV	850ppm QAC concentration of R-82 formulation surface disinfection	infectivity assay	~6.5 log reduction	Jimenez and Chiang (2006)
FCV	1000ppm (quaternary dialkyl benzyl ammonium chloride (50%)); 3000ppm(quaternary-alkyl dimethyl benyl ammonium chloride (80%)); surface test (polystyrene Petri dish)	infectivity assay	1000 ppm showed <2.27 log reduction; 3000 showed 1.17 log reduction after 1 minute contact time	Whitehead and McCue (2010)
FCV	food contact surfaces (stainless steel), 450, 900 and 1800ppm of 9% QAC, n-quaternary ammonium comound of alkyl dimethyl benzyl ammonium chloride, 10 minute contact time	infectivity assay	<1 log reduction	Gulati et al. (2001)

Table 1.7 Differentiating Infectious from Inactivated Virus

Method	Organism	How it works	Point of study and Main findings	Reference
Integrated culture-polymerase chain reaction (C-PCR), RT-PCR, plaque assay; DNA hybridization and nested PCR were used to confirm C-PCR and RT-PCR results	Enteric viruses (poliovirus, ECHOvirus, coxsackie A and B viruses) adenovirus in environmental samples	C-PCR detects presence and identity based on cell culture, CPE, RNA/DNA detection	Evaluate sensitivity, speed and virus infectivity C-PCR was good for detecting enteric viruses in high viral concentration, low toxicity samples, confirmed infectious virus, as sensitive as RT-PCR, results are very rapid	Greening et al (2002)
Nucleic acid amplification and oligonucleotide probing ,	Enteroviruses and hepatitis A in naturally contaminated oysters	RNA isolation of CPE and non CPE cell culture lysates for RT-PCR	Apply and compare nucleic acid amplification with cell culture methods to detect viruses in oysters Detection by RT-PCR increased positive samples by 50% over detection by CPE in cell culture. PCR methods increase detection of noncytopathic human enteric viruses	Chung et al (1996)
Integrated cell culture/ strand specific RT-PCR	Water samples artificially contaminated with infectious or inactivate (by formalin) HAV	Tagged RT-PCR has specificity for HAV (-) strand RNA (replicate intermediate), can distinguish between infectious and non-infectious HAV	Combine RT-PCR and cell culture to differentiate between infectious and noninfectious HAV in spiked water samples ICC/strand-specific RT-PCR allows for rapid detection of infectious HAV in clinical, environmental and food samples	Jiang et al (2004)

Table 1.7 cont.)

Method	Organism	How it works	Point of study and Main findings	Reference
Proteinase K, RNase pretreatment, inactivation by: UV, heat, hypochlorite	Human picornavirus, feline calicivirus	Proteinase will digest damaged capsid protein, RNase will digest exposed RNA to not have false positives	Demonstrate functional changes in capsid after inacivation Virus inactivation correlates with loss of virus ability to attach to host cell receptors	Nuanualsuwan and Cliver (2003)
UV, heat, hypochlorite inactivation, pretreatment with proteinase K and RNase prior to RT-PCR; infectivity assay	HAV, poliovirus, FCV		Mixing RNase with proteinase K to add to the mixture prior to RT-PCR was best, FCV may not be good surrogate, pretreatment before RT-PCR was successful in preventing inactivated virus	Nuanualsuwan and Cliver (2002)
Persistence on refrigerated RTE food; NASBA; infectivity assay; pretreatment with proteinase K and RNase	FCV, NoV		FCV heat and enzymatic pretreatment sensitive than NoV, NoV survived at least 10 days on RTE food,	Lamhoujeb et al (2008)
Magnetic bead-based HBGA assay, RNase pretreatment	NoV	NoV recognize HBGA receptors, attach to receptors that are attached to magnetic beads, viral RNA is then detected after washes	HBGA magnetic bead separation method worked to detect NoV with an intact HBGA receptor site	Cannon and Vinje (2008)

Table 1.7 cont.)

Method	Organism	How it works	Point of study and Main findings	Reference
ELISA, RT-PCR Immunomagnetic beads used to capture and concentrate the virus from contaminated foods prior to RT-PCR to detect NoV	NoV from implicated food in non-oyster outbreak		Immunomagnetic capture RT-PCR shown to be rapid and simple for NoV recovery from foods and identification of NoV	Kobayashi et al (2004)
Paramagnetic beads with goat anti rabit IgG beads	NoV from stool, HAV		Concentrated virus (2000 fold compared to direct heat release), partial virus purification	Gilpatrick et al (2000)
DNA enzyme immunoassay-capture PCR product by biotinylated probes on streptavidin coated wells	NoV in oysters		Low levels of NoV were detected using this method, decrease in time and able to be automated	Schwab et al (2001)
DNA aptamers	*Salmonella enterica* serovars: *S. Ttyphimurium*	Single stranded oligonucleotides which fold into 3D structures and can bind to specific surfaces	Capture and concentrate low levels of bacteria from a food matrix Aptamers can be used to capture whole bacterial cells	Joshi et al (2009)

Table 1.7 cont.)

Method	Organism	How it works	Point of study and Main findings	Reference
PMA, qPCR, *E. coli,* moderate heat, protease treatment	Bacteriophage T4	PMA penetrates virus with damaged capsid,	PMA penetrates virus with damaged capsid, may not be relevant if infectivity is lost due to cell receptor and not compromised capsid; PMA pre-treatment could distinguish between infectious virus in cases with significant damage to viral capsid integrity	Fittipaldi et al (2010)
PMA with RT-PCR	Coxasckievirus, poliovirus, echovirus, and Norwalk virus inactivated by heat or hypochlorite		PMA treatment did not interfere with detection of infectious, did prevent noninfectious or inactivated virus from being detected	Parshionikar et al (2010)
Cell culture with Caco-2 and insect Spodoptera frugiperda 9; testing VLP attachment	Recombinant Norwalk virus like particles; Caco- 2 cells		Use recombinant Norwalk virus like particles to study binding and internalization of VLP to human and animal cell line to try to understand infectivity of NoV C-terminal region of capsid protein is involved in binding of rNV VLPs to cells	White et al (1996)
Sodium hypochlorite treatment, biotinylation and avidin immobilized affinity chromatography, RT-PCR,	Human astrovirus type 4, Caco-2, NoV GII.4,		Oxidative products on viral capsid may be quantified to detect virus integrity (correlates with infectivity)	Sano et al (2010)

Table 1.7 cont.)

Method	Organism	How it works	Point of study and Main findings	Reference
Cell culture, heat and UV light inactivation; duplex RT-qPCR: short range RT-qPCR, long range RT-qPCR	MNV-1, HuNoV GI and GII		Long range RT-qPCR correlated with loss of infectivity as determined by cell culture (MNV); direct linear correlation between fragment size and rate of RNA degradation	Wolf et al (2009)
Photosensitized plasmid RNA, use of oxidation of dyes			AMV reverse transcriptase is useful in detecting modified bases; possible to survey the amount of cellular RNA damage	Rhee et al (1995)

1.9 REFERENCES

1. Alvarez, M. E., and R. T. O'Brien. 1982. Mechanisms of inactivation of poliovirus by chlorine dioxide and iodine. Appl Environ Microbiol 44:1064-71.

2. Atmar, R. L., A. R. Opekun, M. A. Gilger, M. K. Estes, S. E. Crawford, F. H. Neill, and D. Y. Graham. 2008. Norwalk virus shedding after experimental human infection. Emerg Infect Dis 14:1553-7.

3. Baert, L., I. Vandekinderen, F. Devlieghere, E. Van Coillie, J. Debevere, and M. Uyttendaele. 2009. Efficacy of sodium hypochlorite and peroxyacetic acid to reduce murine norovirus 1, B40-8, Listeria monocytogenes, and Escherichia coli O157:H7 on shredded iceberg lettuce and in residual wash water. J Food Prot 72:1047-54.

4. Baert, L., C. E. Wobus, E. Van Coillie, L. B. Thackray, J. Debevere, and M. Uyttendaele. 2008. Detection of murine norovirus 1 by using plaque assay, transfection assay, and real-time reverse transcription-PCR before and after heat exposure. Appl Environ Microbiol 74:543-6.

5. Balogh, Z., G. Lautner, V. Bardoczy, B. Komorowska, R. E. Gyurcsanyi, and T. Meszaros. Selection and versatile application of virus-specific aptamers. Faseb J 24:4187-95.

6. Barker, J., I. B. Vipond, and S. F. Bloomfield. 2004. Effects of cleaning and disinfection in reducing the spread of Norovirus contamination via environmental surfaces. J Hosp Infect 58:42-9.

7. Bean, N. H., J. S. Goulding, C. Lao, and F. J. Angulo. 1996. Surveillance for foodborne-disease outbreaks--United States, 1988-1992. MMWR CDC Surveill Summ 45:1-66.

8. Belliot, G., A. Lavaux, D. Souihel, D. Agnello, and P. Pothier. 2008. Use of murine norovirus as a surrogate to evaluate resistance of human norovirus to disinfectants. Appl Environ Microbiol 74:3315-8.

9. Bertolotti-Ciarlet, A., L. J. White, R. Chen, B. V. Prasad, and M. K. Estes. 2002. Structural requirements for the assembly of Norwalk virus-like particles. J Virol 76:4044-55.

10. Bidawid, S., J. M. Farber, and S. A. Sattar. 2000. Inactivation of hepatitis A virus (HAV) in fruits and vegetables by gamma irradiation. International Journal of Food Microbiology 57:91-97.

11. Blackmer, F., K. A. Reynolds, C. P. Gerba, and I. L. Pepper. 2000. Use of integrated cell culture-PCR to evaluate the effectiveness of poliovirus inactivation by chlorine. Appl Environ Microbiol 66:2267-8.

12. Buckow, R., S. Isbarn, D. Knorr, V. Heinz, and A. Lehmacher. 2008. Predictive model for inactivation of feline calicivirus, a norovirus surrogate, by heat and high hydrostatic pressure. Appl Environ Microbiol 74:1030-8.

13. Bull, R. A., J. S. Eden, W. D. Rawlinson, and P. A. White. Rapid evolution of pandemic noroviruses of the GII.4 lineage. PLoS Pathog 6:e1000831.

14. Cannon, J. L., E. Papafragkou, G. W. Park, J. Osborne, L. A. Jaykus, and J. Vinje. 2006. Surrogates for the study of norovirus stability and inactivation in the environment: aA comparison of murine norovirus and feline calicivirus. J Food Prot 69:2761-5.

15. Cannon, J. L., and J. Vinje. 2008. Histo-blood group antigen assay for detecting noroviruses in water. Appl Environ Microbiol 74:6818-9.

16. Chaidez, C., M. Moreno, W. Rubio, M. Angulo, and B. Valdez. 2003. Comparison of the disinfection efficacy of chlorine-based products for inactivation of viral indicators and pathogenic bacteria in produce wash water. Int J Environ Health Res 13:295-302.

17. Cheesbrough, J. S., L. Barkess-Jones, and D. W. Brown. 1997. Possible prolonged environmental survival of small round structured viruses. J Hosp Infect 35:325-6.

18. Chen, H., D. G. Hoover, and D. H. Kingsley. 2005. Temperature and treatment time influence high hydrostatic pressure inactivation of feline calicivirus, a norovirus surrogate. J Food Prot 68:2389-94.

19. Choi, S., and S. C. Jiang. 2005. Real-time PCR quantification of human adenoviruses in urban rivers indicates genome prevalence but low infectivity. Appl Environ Microbiol 71:7426-33.

20. Chung, H., L. A. Jaykus, and M. D. Sobsey. 1996. Detection of human enteric viruses in oysters by in vivo and in vitro amplification of nucleic acids. Appl Environ Microbiol 62:3772-8.

21. Clarke, I. N., and P. R. Lambden. 2000. Organization and expression of calicivirus genes. J Infect Dis 181 Suppl 2:S309-16.

22. Clasen, T., I. Roberts, T. Rabie, W. Schmidt, and S. Cairncross. 2006. Interventions to improve water quality for preventing diarrhoea. Cochrane Database Syst Rev 3:CD004794.

23. Cromeans, T. L., A. M. Kahler, and V. R. Hill. 2010. Inactivation of adenoviruses, enteroviruses, and murine norovirus in water by free chlorine and monochloramine. Appl Environ Microbiol 76:1028-33.

24. D'Souza, D. H., A. Sair, K. Williams, E. Papafragkou, J. Jean, C. Moore, and L. Jaykus. 2006. Persistence of caliciviruses on environmental surfaces and their transfer to food. Int J Food Microbiol 108:84-91.

25. De Gusseme, B., L. Sintubin, L. Baert, E. Thibo, T. Hennebel, G. Vermeulen, M. Uyttendaele, W. Verstraete, and N. Boon. 2010. Biogenic silver for disinfection of water contaminated with viruses. Appl Environ Microbiol 76:1082-7.

26. Deborde, M., and U. von Gunten. 2008. Reactions of chlorine with inorganic and organic compounds during water treatment-Kinetics and mechanisms: a critical review. Water Res 42:13-51.

27. Donaldson, E. F., L. C. Lindesmith, A. D. Lobue, and R. S. Baric. 2008. Norovirus pathogenesis: mechanisms of persistence and immune evasion in human populations. Immunol Rev 225:190-211.

28. Doultree, J. C., J. D. Druce, C. J. Birch, D. S. Bowden, and J. A. Marshall. 1999. Inactivation of feline calicivirus, a Norwalk virus surrogate. J Hosp Infect 41:51-7.

29. Duizer, E., P. Bijkerk, B. Rockx, A. De Groot, F. Twisk, and M. Koopmans. 2004. Inactivation of caliciviruses. Appl Environ Microbiol 70:4538-43.

30. Espinosa, A. C., M. Mazari-Hiriart, R. Espinosa, L. Maruri-Avidal, E. Mendez, and C. F. Arias. 2008. Infectivity and genome persistence of rotavirus and astrovirus in groundwater and surface water. Water Res 42:2618-28.

31. Fastier, L. B. 1957. A new feline virus isolated in tissue culture. Am J Vet Res 18:382-9.

32. Flint S.J, E. L. W., Racaniello V.R., Skalka A.M. 2003. Principles of Virology: Molecular Biology, Pathogenesis, and Control of Animal Viruses, second ed.

33. Fong, T. T., and E. K. Lipp. 2005. Enteric viruses of humans and animals in aquatic environments: health risks, detection, and potential water quality assessment tools. Microbiol Mol Biol Rev 69:357-71.

34. Gehrke, C., J. Steinmann, and P. Goroncy-Bermes. 2004. Inactivation of feline calicivirus, a surrogate of norovirus (formerly Norwalk-like viruses), by different types of alcohol in vitro and in vivo. J Hosp Infect 56:49-55.

35. Gilpatrick, S. G., K. J. Schwab, M. K. Estes, and R. L. Atmar. 2000. Development of an immunomagnetic capture reverse transcription-PCR assay for the detection of Norwalk virus. J Virol Methods 90:69-78.

36. Girard, M., S. Ngazoa, K. Mattison, and J. Jean. 2010. Attachment of noroviruses to stainless steel and their inactivation, using household disinfectants. J Food Prot 73:400-4.

37. Glass, R. I., U. D. Parashar, and M. K. Estes. 2009. Norovirus gastroenteritis. N Engl J Med 361:1776-85.

38. Gulati, B. R., P. B. Allwood, C. W. Hedberg, and S. M. Goyal. 2001. Efficacy of commonly used disinfectants for the inactivation of calicivirus on strawberry, lettuce, and a food-contact surface. J Food Prot 64:1430-4.

39. Harrington, P. R., L. Lindesmith, B. Yount, C. L. Moe, and R. S. Baric. 2002. Binding of Norwalk virus-like particles to ABH histo-blood group antigens is blocked by antisera from infected human volunteers or experimentally vaccinated mice. J Virol 76:12335-43.

40. Helentjaris, T., and E. Ehrenfeld. 1977. Inhibition of host cell protein synthesis by UV-inactivated poliovirus. J Virol 21:259-67.

41. Hijnen, W. A., E. F. Beerendonk, and G. J. Medema. 2006. Inactivation credit of UV radiation for viruses, bacteria and protozoan (oo)cysts in water: a review. Water Res 40:3-22.

42. Hudson, J. B., M. Sharma, and M. Petric. 2007. Inactivation of Norovirus by ozone gas in conditions relevant to healthcare. J Hosp Infect 66:40-5.

43. Jiang, X., M. Wang, K. Wang, and M. K. Estes. 1993. Sequence and genomic organization of Norwalk virus. Virology 195:51-61.

44. Jiang, Y. J., G. Y. Liao, W. Zhao, M. B. Sun, Y. Qian, C. X. Bian, and S. D. Jiang. 2004. Detection of infectious hepatitis A virus by integrated cell culture/strand-specific reverse transcriptase-polymerase chain reaction. J Appl Microbiol 97:1105-12.

45. Jimenez, L., and M. Chiang. 2006. Virucidal activity of a quaternary ammonium compound disinfectant against feline calicivirus: A surrogate for norovirus. Am J Infect Control 34:269-73.

46. Jothikumar, N., D. O. Cliver, and T. W. Mariam. 1998. Immunomagnetic capture PCR for rapid concentration and detection of hepatitis A virus from environmental samples. Appl Environ Microbiol 64:504-8.

47. Kapikian, A. Z., R. G. Wyatt, R. Dolin, T. S. Thornhill, A. R. Kalica, and R. M. Chanock. 1972. Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. J Virol 10:1075-81.

48. Karst, S. M., C. E. Wobus, M. Lay, J. Davidson, and H. W. t. Virgin. 2003. STAT1-dependent innate immunity to a Norwalk-like virus. Science 299:1575-8.

49. Keswick, B. H., T. K. Satterwhite, P. C. Johnson, H. L. DuPont, S. L. Secor, J. A. Bitsura, G. W. Gary, and J. C. Hoff. 1985. Inactivation of Norwalk virus in drinking water by chlorine. Appl Environ Microbiol 50:261-4.

50. Kim, C. K., D. M. Gentile, and O. J. Sproul. 1980. Mechanism of Ozone Inactivation of Bacteriophage f2. Appl Environ Microbiol 39:210-218.

51. Kim, H. Y., I. S. Kwak, I. G. Hwang, and G. Ko. 2008. Optimization of methods for detecting norovirus on various fruit. J Virol Methods 153:104-10.

52. Kim, J. G., A. E. Yousef, and S. Dave. 1999. Application of ozone for enhancing the microbiological safety and quality of foods: a review. J Food Prot 62:1071-87.

53. Kingsley, D. H., D. R. Holliman, K. R. Calci, H. Chen, and G. J. Flick. 2007. Inactivation of a norovirus by high-pressure processing. Appl Environ Microbiol 73:581-5.

54. Kingsley, D. H., D. G. Hoover, E. Papafragkou, and G. P. Richards. 2002. Inactivation of hepatitis A virus and a calicivirus by high hydrostatic pressure. J Food Prot 65:1605-9.

55. Kitajima, M., Y. Tohya, K. Matsubara, E. Haramoto, E. Utagawa, and H. Katayama. 2010. Chlorine inactivation of human norovirus, murine norovirus and poliovirus in drinking water. Lett Appl Microbiol 51:119-21.

56. Kobayashi, S., K. Natori, N. Takeda, and K. Sakae. 2004. Immunomagnetic capture rt-PCR for detection of norovirus from foods implicated in a foodborne outbreak. Microbiol Immunol 48:201-4.

57. Lages, S. L., M. A. Ramakrishnan, and S. M. Goyal. 2008. In-vivo efficacy of hand sanitisers against feline calicivirus: a surrogate for norovirus. J Hosp Infect 68:159-63.

58. Lamhoujeb, S., H. Charest, I. Fliss, S. Ngazoa, and J. Jean. 2009. Real-time molecular beacon NASBA for rapid and sensitive detection of norovirus GII in clinical samples. Can J Microbiol 55:1375-80.

59. Lamhoujeb, S., I. Fliss, S. E. Ngazoa, and J. Jean. 2008. Evaluation of the persistence of infectious human noroviruses on food surfaces by using real-time nucleic acid sequence-based amplification. Appl Environ Microbiol 74:3349-55.

60. Lee, J., K. Zoh, and G. Ko. 2008. Inactivation and UV disinfection of murine norovirus with TiO2 under various environmental conditions. Appl Environ Microbiol 74:2111-7.

61. Li, J. W., Z. T. Xin, X. W. Wang, J. L. Zheng, and F. H. Chao. 2002. Mechanisms of inactivation of hepatitis a virus by chlorine. Appl Environ Microbiol 68:4951-5.

62. Li, J. W., Z. T. Xin, X. W. Wang, J. L. Zheng, and F. H. Chao. 2004. Mechanisms of inactivation of hepatitis A virus in water by chlorine dioxide. Water Res 38:1514-9.

63. Lim, M. Y., J. M. Kim, J. E. Lee, and G. Ko. Characterization of ozone disinfection of murine norovirus. Appl Environ Microbiol 76:1120-4.

64. Lindesmith, L., C. Moe, J. Lependu, J. A. Frelinger, J. Treanor, and R. S. Baric. 2005. Cellular and humoral immunity following Snow Mountain virus challenge. J Virol 79:2900-9.

65. Liu, B., P. Maywood, L. Gupta, and B. Campbell. 2003. An outbreak of Norwalk-like virus gastroenteritis in an aged-care residential hostel. N S W Public Health Bull 14:105-9.

66. Liu, P., Chien, Y., Papafragkou, E., Hsiao H., Jaykus, L., Moe, C. 2009. Persistence of Human Noroviruses on Food Preparation Surfaces and Human Hands. Food Environment Virology 1:141-147.

67. Liu, P., Y. Yuen, H. M. Hsiao, L. A. Jaykus, and C. Moe. 2010. Effectiveness of liquid soap and hand sanitizer against Norwalk virus on contaminated hands. Appl Environ Microbiol 76:394-9.

68. Lopman, B. A., D. W. Brown, and M. Koopmans. 2002. Human caliciviruses in Europe. J Clin Virol 24:137-60.

69. Macinga, D. R., S. A. Sattar, L. A. Jaykus, and J. W. Arbogast. 2008. Improved inactivation of nonenveloped enteric viruses and their surrogates by a novel alcohol-based hand sanitizer. Appl Environ Microbiol 74:5047-52.

70. Malik, Y. S., and S. M. Goyal. 2006. Virucidal efficacy of sodium bicarbonate on a food contact surface against feline calicivirus, a norovirus surrogate. Int J Food Microbiol 109:160-3.

71. Malik, Y. S., S. Maherchandani, and S. M. Goyal. 2006. Comparative efficacy of ethanol and isopropanol against feline calicivirus, a norovirus surrogate. Am J Infect Control 34:31-5.

72. Mallet, J. C., Beghian, L.E., Metcalf, T.G., and Kaylor, J.D. 1991. Potential of Irradiation technology for improving shellfish sanitation. Journal of Food Safety 11:231-245.

73. Marks, P. J., I. B. Vipond, D. Carlisle, D. Deakin, R. E. Fey, and E. O. Caul. 2000. Evidence for airborne transmission of Norwalk-like virus (NLV) in a hotel restaurant. Epidemiol Infect 124:481-7.

74. Mattison, K., K. Karthikeyan, M. Abebe, N. Malik, S. A. Sattar, J. M. Farber, and S. Bidawid. 2007. Survival of calicivirus in foods and on surfaces: experiments with feline calicivirus as a surrogate for norovirus. J Food Prot 70:500-3.

75. Mbithi, J. N., V. S. Springthorpe, and S. A. Sattar. 1993. Comparative in vivo efficiencies of hand-washing agents against hepatitis A virus (HM-175) and poliovirus type 1 (Sabin). Appl Environ Microbiol 59:3463-9.

76. McDonnell, G., and A. D. Russell. 1999. Antiseptics and disinfectants: activity, action, and resistance. Clin Microbiol Rev 12:147-79.

77. McKillip, J. L., L. A. Jaykus, and M. Drake. 1998. rRNA stability in heat-killed and UV-irradiated enterotoxigenic Staphylococcus aureus and Escherichia coli O157:H7. Appl Environ Microbiol 64:4264-8.

78. Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. Emerg Infect Dis 5:607-25.

79. Moe, C. L. 2009. Preventing norovirus transmission: how should we handle food handlers? Clin Infect Dis 48:38-40.

80. Morton, V., J. Jean, J. Farber, and K. Mattison. 2009. Detection of noroviruses in ready-to-eat foods by using carbohydrate-coated magnetic beads. Appl Environ Microbiol 75:4641-3.

81. Nocker, A., C. Y. Cheung, and A. K. Camper. 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J Microbiol Methods 67:310-20.

82. Nuanualsuwan, S., and D. O. Cliver. 2003. Capsid functions of inactivated human picornaviruses and feline calicivirus. Appl Environ Microbiol 69:350-7.

83. Nuanualsuwan, S., and D. O. Cliver. 2002. Pretreatment to avoid positive RT-PCR results with inactivated viruses. J Virol Methods 104:217-25.

84. O'Brien, R. T., and J. Newman. 1979. Structural and compositional changes associated with chlorine inactivation of polioviruses. Appl Environ Microbiol 38:1034-9.

85. Park, G. W., D. M. Boston, J. A. Kase, M. N. Sampson, and M. D. Sobsey. 2007. Evaluation of liquid- and fog-based application of Sterilox hypochlorous acid solution for surface inactivation of human norovirus. Appl Environ Microbiol 73:4463-8.

86. Park, Y., Y. H. Cho, Y. Jee, and G. Ko. 2008. Immunomagnetic separation combined with real-time reverse transcriptase PCR assays for detection of norovirus in contaminated food. Appl Environ Microbiol 74:4226-30.

87. Parshionikar, S., I. Laseke, and G. S. Fout. 2010. Use of propidium monoazide in reverse transcriptase PCR to distinguish between infectious and noninfectious enteric viruses in water samples. Appl Environ Microbiol 76:4318-26.

88. Pesavento, P. A., K. O. Chang, and J. S. Parker. 2008. Molecular virology of feline calicivirus. Vet Clin North Am Small Anim Pract 38:775-86, vii.

89. Poschetto, L. F., A. Ike, T. Papp, U. Mohn, R. Bohm, and R. E. Marschang. 2007. Comparison of the sensitivities of noroviruses and feline calicivirus to chemical disinfection under field-like conditions. Appl Environ Microbiol 73:5494-500.

90. Roy, D., P. K. Wong, R. S. Engelbrecht, and E. S. Chian. 1981. Mechanism of enteroviral inactivation by ozone. Appl Environ Microbiol 41:718-23.

91. Russell, A. D. 2004. Whither triclosan? J Antimicrob Chemother 53:693-5.

92. Scallan, E., P. M. Griffin, F. J. Angulo, R. V. Tauxe, and R. M. Hoekstra. Foodborne illness acquired in the United States--unspecified agents. Emerg Infect Dis 17:16-22.

93. Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis 17:7-15.

94. Schwab, K. J., R. De Leon, and M. D. Sobsey. 1996. Immunoaffinity concentration and purification of waterborne enteric viruses for detection by reverse transcriptase PCR. Appl Environ Microbiol 62:2086-94.

95. Scipioni, A., A. Mauroy, J. Vinje, and E. Thiry. 2008. Animal noroviruses. Vet J.

96. Shieh, Y. C., C. I. Wong, J. A. Krantz, and F. C. Hsu. 2008. Detection of naturally occurring enteroviruses in waters using direct RT-PCR and integrated cell culture-RT-PCR. J Virol Methods 149:184-9.

97. Shin, G. A., and M. D. Sobsey. 2003. Reduction of Norwalk virus, poliovirus 1, and bacteriophage MS2 by ozone disinfection of water. Appl Environ Microbiol 69:3975-8.

98. Simonet, J., and C. Gantzer. 2006. Degradation of the Poliovirus 1 genome by chlorine dioxide. J Appl Microbiol 100:862-70.

99. Sintubin, L., W. De Windt, J. Dick, J. Mast, D. van der Ha, W. Verstraete, and N. Boon. 2009. Lactic acid bacteria as reducing and capping agent for the fast and efficient production of silver nanoparticles. Appl Microbiol Biotechnol 84:741-9.

100. Slomka, M. J., and H. Appleton. 1998. Feline calicivirus as a model system for heat inactivation studies of small round structured viruses in shellfish. Epidemiol Infect 121:401-7.

101. Studdert, M. J. 1978. Caliciviruses. Brief review. Arch Virol 58:157-91.

102. Teunis, P. F., C. L. Moe, P. Liu, S. E. Miller, L. Lindesmith, R. S. Baric, J. Le Pendu, and R. L. Calderon. 2008. Norwalk virus: how infectious is it? J Med Virol 80:1468-76.

103. Thurston-Enriquez, J. A., C. N. Haas, J. Jacangelo, K. Riley, and C. P. Gerba. 2003. Inactivation of feline calicivirus and adenovirus type 40 by UV radiation. Appl Environ Microbiol 69:577-82.

104. Todd, E. C., J. D. Greig, C. A. Bartleson, and B. S. Michaels. 2009. Outbreaks where food workers have been implicated in the spread of foodborne disease. Part 6. Transmission and survival of pathogens in the food processing and preparation environment. J Food Prot 72:202-19.

105. Ueki, Y., M. Shoji, A. Suto, T. Tanabe, Y. Okimura, Y. Kikuchi, N. Saito, D. Sano, and T. Omura. 2007. Persistence of caliciviruses in artificially contaminated oysters during depuration. Appl Environ Microbiol 73:5698-701.

106. Urakami, H., K. Ikarashi, K. Okamoto, Y. Abe, T. Ikarashi, T. Kono, Y. Konagaya, and N. Tanaka. 2007. Chlorine sensitivity of feline calicivirus, a norovirus surrogate. Appl Environ Microbiol 73:5679-82.

107. Wetz, J. J., E. K. Lipp, D. W. Griffin, J. Lukasik, D. Wait, M. D. Sobsey, T. M. Scott, and J. B. Rose. 2004. Presence, infectivity, and stability of enteric viruses in seawater: relationship to marine water quality in the Florida Keys. Mar Pollut Bull 48:698-704.

108. Whitehead, K., and K. A. McCue. 2010. Virucidal efficacy of disinfectant actives against feline calicivirus, a surrogate for norovirus, in a short contact time. Am J Infect Control 38:26-30.

109. Widdowson, M. A., A. Sulka, S. N. Bulens, R. S. Beard, S. S. Chaves, R. Hammond, E. D. Salehi, E. Swanson, J. Totaro, R. Woron, P. S. Mead, J. S. Bresee, S. S. Monroe, and R. I. Glass. 2005. Norovirus and foodborne disease, United States, 1991-2000. Emerg Infect Dis 11:95-102.

110. Wobus, C. E., L. B. Thackray, and H. W. t. Virgin. 2006. Murine norovirus: a model system to study norovirus biology and pathogenesis. J Virol 80:5104-12.

111. Zheng, D. P., T. Ando, R. L. Fankhauser, R. S. Beard, R. I. Glass, and S. S. Monroe. 2006. Norovirus classification and proposed strain nomenclature. Virology 346:312-23.

CHAPTER TWO

EFFICACY OF COMMONLY USED DISINFECTANTS FOR INACTIVATION OF HUMAN NOROVIRUSES AND ITS SURROGATES

2.1. ABSTRACT

Human noroviruses (HuNoV) are the leading cause of food borne disease. There is a need to better characterize the efficacy of standard disinfectants, and elucidate the relationship between virus infectivity and molecular detection for HuNoV and their cultivable surrogates. The purpose of this study was to compare the efficacy of three commonly used disinfectants against representative HuNoV strains and cultivable surrogates using quantitative reverse transcription PCR (RT-qPCR) and infectivity assay. Ethanol (50%, 70% and 90%), sodium hypochlorite (5, 75, 250, 500 and 1,000 ppm) and a quaternary ammonium compound (QUAT, at 0.1X, 1.0X, and 10X concentrations) were evaluated against two HuNoV genogroup II strains (GII.2 and GII.4) and two surrogates [feline calicivirus (FCV) and murine norovirus (MNV-1)]. Virucidal suspension assays (30-sec exposure) were conducted in accordance with ASTM E-1052. Virus inactivation was quantified using RT-qPCR targeting the *orfI-orfII* junction (HuNoV), the RNA polymerase region (MNV-1), or the *orf* I region (FCV); infectivity assays were also performed for MNV-1 and FCV. Log reductions of 0.6, 2.3 and 2.3 were observed by RT-qPCR for MNV-1 after 30 sec exposures to 50, 70 and 90% ethanol, respectively. By infectivity assay, these numbers were 0.3, 3.5 and >3.5. For FCV,

HuNoV GII.2 and GII.4 strains, there was no statistically significant ($p<0.05$) reduction in virus titer, regardless of ethanol concentration, by either RT-qPCR or infectivity assay. By RT-qPCR, HuNoV GII.2 was completely resistant to chlorine concentrations ≤1,000 ppm; GII.4 showed a 4.5 log reduction at 1,000 ppm chlorine but no significant inactivation at concentrations ≤500 ppm. MNV-1 and FCV were much more susceptible to chlorine, demonstrating a 3.0 log reduction at concentrations ranging from 250-500 ppm. Log reductions based on RT-pPCR and infectivity assay (MNV-1 and FCV) correlated well for chlorine treatments. The QUAT compound was ineffective against all four viruses (<0.5 $\log_{10}$ reduction) at all concentrations tested. This study demonstrates the relative ineffectiveness of disinfectants against HuNoV and highlights the fact that behavior of the cultivable surrogates does not always mimic that of human strains.

2.2. INTRODUCTION

Food borne illness is a major cause of morbidity and mortality worldwide, with 9.4 million cases, about 56,000 hospitalizations, and 1,351 deaths caused by 31 known pathogens in the U.S. alone (39).When also taking into account cases associated with unspecified agents, the numbers are much higher, with a combined total of 47.8 million episodes per year (38). Similar to earlier reports by Mead et al. (1999), Scallan et al. (2011a, b) reiterate the importance of viruses, which are now estimated to cause 59% of food borne disease cases (31). From an epidemiological standpoint, the agents responsible for the majority of acute viral gastroenteritis are the HuNoVs, these viruses

also cause most viral food borne illnesses of known etiology, constituting between 3.3 and 8.3 million cases per year (39).

HuNoV causes a disease characterized by vomiting (hallmark symptom), nausea, diarrhea, abdominal pain, and sometimes headache and low grade fever. The illness is usually self-limiting, but on occasion will require hospitalization to treat dehydration. By virtue of the sheer numbers of food borne HuNoV cases per year, even a low likelihood of severe disease (0.03%) or death will still result in a large number of serious outcomes. Current estimates are that HuNoVs are responsible for 15,000 hospitalizations and 1500 deaths annually in the U.S. (39). Even these figures may be considered an underestimate because the disease is difficult to diagnose and cases frequently go undetected or outbreaks, uninvestigated.

HuNoV are transmitted by the fecal-oral route, and viruses can be shed in high numbers (10^8 genome copies or more per g) (24) and for extended periods of time (three weeks) (37) in the feces of infected individuals. More recently, it has become clear that HuNoV are released in vomitus, and it is now suspected that aerosol formation after projectile vomiting may also be an important transmission route (6). Because of these two transmission routes, as well as their notably low infectious dose (ranging from 10-100 virus particles) (43) and the transient immunity of the host (35), HuNoV are considered highly transmissible. In the food processing and preparation sector, this is exacerbated by the fact that HuNoV persist in the environment for extended periods of time, as evidenced by prolonged outbreaks and laboratory-based studies (5, 16) .

Humans are the sole host for almost all members of the Norovirus genus and human strains cannot be cultivated *in vitro*. In the early days of their identification, HuNoV could only be identified using electron microscopy. This changed about two decades ago with the introduction of molecular amplification methods, specifically reverse transcription polymerase chain reaction (RT-PCR). While a major step forward, we now know that the RNA of viral genomes can persist long after inactivation of the virus particle (7, 11, 41). Hence, although a positive detection signal by RT-PCR may indicate the presence of viral RNA, it cannot assure that infectious virus is present in the sample.

In the absence of *in vitro* cultivation methods for HuNoV, investigators have relied on cultivable surrogates to study the behavior of these viruses. The two surrogates most often used are feline calicivirus (FCV) and murine norovirus (MNV-1). FCV belongs to one of the other genera in the *Caliciviridea* family and is easy to culture; however, its genome varies significantly from that of HuNoV, it is transmitted by the respiratory rather than the fecal-oral route, and perhaps most importantly, FCV is much more sensitive to extremes of pH than are HuNoV (3, 8, 44). On the other hand, MNV-1 shares more likeness to HuNoV with respect to biochemical and genetic features, and is also transmitted by the fecal-oral route (47). However, there are differences. Specifically, MNV-1 does not bind with histoblood group antigens (HBGAs), important cofactors for HuNoV infection (47); it causes a neurological rather than gastrointestinal infection in mice (21); and it appears to be more sensitive than to dessication (3). The current

scientific consensus is that, although useful, the behavior of the surrogates may not always be representative of that of HuNoV.

This has important ramifications in evaluating the efficacy of surface and hand disinfectants, both of which are important components in controlling the transmission of food borne pathogens. While many disinfectants are used across the farm-to-fork continuum, the most common are sodium hypochlorite, ethanol, and quaternary ammonium compounds. Sodium hypochlorite is the active compound of bleach; it is a strong oxidizing agents and used predominantly for surface disinfection (46). Undiluted household cleaners can contain concentrations as high as 5,000 ppm chlorine, but regulatory limits for the use of hypochlorite on food contact surfaces is 200 ppm (21 CFR Part 178). Higher concentrations may be used, but only if followed by a potable water rinse. Quaternary ammonium compounds at a use concentration of 200 ppm are also commonly used for surface decontamination (18). Alcohols (ethanol and isopropanol) at concentrations ranging from 60-95% constitute the active agent in many commercial hand sanitizers (12).

Most studies undertaken to evaluate the efficacy of disinfectants against HuNoV have been applied either solely to human strains using RT-PCR to measure virus inactivation, or else to surrogates whose survival was assessed exclusively by infectivity assay. Very few studies have included RT-PCR with infectivity assay (for surrogates) or have compared surrogates with human strains. However, such comparative studies provide valuable information on the relevance of the surrogates, and/or on the

relationship between infectivity and molecular amplification. This following study was designed to provide such information. Specifically, we compared the disinfection efficacy of three commonly used disinfectants (ethanol, hypochlorite, and quaternary ammonium) at multiple concentrations, against HuNoV and two cultivable surrogates (FCV and MNV-1), using a combination of cell culture infectivity assay and quantitative RT-PCR (RT-qPCR). The experimental design and subsequent statistical analysis allowed us to assess three major relationships: (i) the effect of disinfectant concentration on virus inactivation; (ii) the relationship between disinfectant efficacy as evaluated by RT-qPCR and infectivity assay; and (iii) a comparison of the efficacy of disinfectants as applied to HuNoV and their commonly used cultivable surrogates.

2.3. MATERIALS AND METHODS

2.3.1. Viruses, mammalian cell lines, and virus propagation

Fecal specimens containing Snow Mountain virus (GII.2, courtesy of Dr. Christine Moe, Emory University, Atlanta, GA) and the epidemic GII.4 strain (courtesy of Dr. Shermalyn Greene, NC Department of Heath and Human Services), obtained from human challenge studies and outbreaks, respectively, were used as representative HuNoV strains. These were suspended 20% in phosphate buffered saline (PBS) and had relatively high titer [10^4 RT-qPCR amplifiable units (PCRU)/µl (corresponding to approx. 10^7 PCRU/ml)]. HuNoV stock solutions were aliquoted and stored at -80°C until use.

Murine norovirus (MNV-1) strain P3 (obtained courtesy of Dr. Howard Virgin, Washington University, St. Louis, MO) was propagated in RAW 264.7 (TIB-71) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (ATCC) supplemented with 10% low endotoxin fetal bovine serum (HyClone, Logan, Utah). Feline calicivirus (FCV strain F9, ATCC VR-782) was propagated on CRFK (Crandell Reese Feline Kidney) cells (CCL-94) (ATCC, Manassas, VA) cultured in complete Dulbecco's modified Eagle's medium (ATCC) also supplemented with 10% low endotoxin fetal bovine serum (HyClone). Cells were grown to 80-90% confluence prior to virus infection. Stock solutions of MNV-1 and FCV consisted of cell culture lysates obtained by sequential (3X) freeze-thaw of infected cells showing complete (100%) cytopathic effects. The titer of MNV-1 and FCV stocks ranged from 10^7- 10^8 PFU/ml. Stock solutions were aliquoted and stored at -80°C until use. Standard plaque assay techniques were used to determine infectious titer of MNV-1 and FCV in accordance with the methods of Wobus et al. (2004) and Bidawid et al (2003), respectively (Figure 2.1 and 2.2).

2.3.2 Disinfectants and Antiseptics

Ethanol solutions (50%, 70% and 90%) were made by dilution of 100% ethanol (Pharmaco-Aaper, Brookfield, CT) in RNase/DNase free deionized water. Hypochlorite (bleach) stocks (5 ppm, 75 ppm, 250 ppm, 500 ppm, and 1,000 ppm) were prepared by

first diluting commercial bleach in sodium phosphate buffer, pH 7.3 with further dilution in RNase/DNase-free deionized water and subsequent pH adjustment to 7.0-7.5. Total chlorine concentration was confirmed using the HACH (Loveland, Colorado) Digital Titrator Model 16900. BARDAC® 208M Blend [consisting of 20% alkyl (C_{14} 50%, C_{12} 40%, C_{16} 10%) dimethyl benzyl ammonium chloride, 15% octyl decyl dimethyl ammonium chloride, 6% dioctyl dimethyl ammonium chloride, 9% didecyl dimethyl ammonium chloride] was used as the QUAT solution. Stock solutions were prepared by diluting commercial powder in deionized water to a 10X concentration followed by serial dilution to 0.1X, 1.0X, followed by a pH adjustment to 6.5.

2.3.3 Disinfection protocol

Disinfection studies were done by suspension assay and in accordance with the *ASTM Standard Test Method for Efficacy of Antimicrobial Agents Against Viruses in Suspension* designation: E 1052-96 (Reapproved, 2002). Briefly, a 25µl volume of virus stock suspension [20% human stool suspension (HuNoV) or clarified cell culture lysate (MNV-1, FCV)] was mixed with 225 µl of disinfectant or antiseptic for a contact time of 30 sec. Twenty µl of this solution was added to 180 µl of fetal bovine serum (FBS) for neutralization, followed by serial dilution and assay (infectivity or RT-qPCR, as appropriate). Consistent with recommendations in the ASTM standard E1052-96, virus, neutralization, and cytotoxicity controls were included in all experiments. Flow diagrams of the experimental protocol are provided in Figures 2.1 and 2.2.

2.3.4 RNA extraction and RT-qPCR

Prior to RT-qPCR, RNA was extracted using the TRIzol reagent (Gibco BRL, Rockville, MD) in accordance with manufacturer instructions. The primer and probe sequences for use in all RT-qPCR assays are shown in Table 2.1. Primer and probe sequences for the GII HuNoV strains corresponded to the conserved *orf*I-*1-orf*II2 junction, as reported by Jothikumar, et al. (2005). Primer and probe sequences for MNV-1 targeted the RNA polymerase region (Dr. Jan Vinje, CDC, personal communication). Beacon Designer 7 (Premier Biosoft International, Palo Alto CA) was used to design the FCV primers and probes based on Genebank ascension number M86379.1, which corresponded to the *orf*1 region of the viral genome.

The *TaqMan* RT-qPCR assay was performed in 25 µl volumes (including 2.5 µl of RNA extract) using the Invitrogen (Carlsbad, California) Superscript III Platinum One-Step Quantitative RT-PCR system and an ABI StepOne real time PCR thermocycler (Applied Biosystems Inc, Foster City, CA). Reverse transcription was done at 50°C for 15 min. After high temperature activation of Hot start DNA polymerase (95°C for 2 min), cDNA amplification followed for a total of 45 cycles of 94°C/10 sec, 54°C/20 sec, and 72°C/15 sec (HuNoV); 95°C/15 sec, 55°C/15 sec, and 72°C/30 sec (MNV-1); or 95°C/ 5 sec and 60°C/ 30 sec (FCV).

For quantification, the resulting Ct values were compared to a standard curve produced by RT-qPCR amplification of 10-fold serially diluted RNA extracted from stock virus. One RT-qPCR amplifiable unit (RT-PCRU) corresponded to the lower limit of detection of this assay. These standard curves are included in the Appendix, Figure 2.10.

2.3.5 Statistical Analysis

Each assay was done in triplicate, and each experiment repeated three times. Log_{10} inactivation was calculated in a manner consistent with the ASTM standard and expressed as mean $\pm$ standard deviation. The Tukey-Kramer multiple comparisons test (GraphPad Instat 3 Program, San Diego, CA) was used in pair-wise comparisons between disinfectants and their concentrations, viruses, and quantification methods (RT-qPCR vs. infectivity assay). Statistical significance was established at the level of $p<0.05$.

2.4. RESULTS

For FCV and both HuNoV strains, $\log_{10}$ reductions were generally <0.5, regardless of ethanol concentration, as evaluated using RT-qPCR (Figure 2.3a). None of these differences were statistically significant. On the other hand, for MNV-1, $\log_{10}$ reductions of 0.6, 2.3, and 2.3 were observed after a 30 sec exposure to 50, 70 and 90% ethanol, respectively. Comparatively, MNV-1 showed $\log_{10}$ inactivation of 0.3, 3.5 and >3.5 as evaluated by infectivity assay (Figure 2.3.b). For both assay types, there was a

statistically significant difference between $\log_{10}$ inactivation for MNV-1 at 50% ethanol versus 70% and 90% ethanol.

Both HuNoV strains were more resistant to hypochlorite than were either animal surrogate, as evaluated using RT-qPCR (Figure 2.4.a). More specifically, for the GII.2 strain, no significant inactivation was observed at hypochlorite concentrations $\leq$1000 ppm; for GII.4, efficacy was only observed at the highest concentration tested (1000 ppm), for which there was a statistically significant reduction in viral genome copy number (4.5 $\log_{10}$, which corresponded to the RT-qPCR assay detection limit). However, for both MNV-1 and FCV, a dose dependent decrease in RNA copies was observed between the ranges of 5 and 500 ppm hypochlorite. More specifically, MNV-1 displayed maximum $\log_{10}$ reductions (3.0, corresponding to the detection limit of the assay) at concentrations of 250 ppm and higher, while FCV showed reductions of 2.2 and 3.0 $\log_{10}$ at 250 and 500 ppm, respectively (again, a 3.0 $\log_{10}$ assay detection limit) although the difference between 250 and 500 ppm was not statistically significant. The dose-dependent hypochlorite inactivation phenomenon was further supported by infectivity assay (Figure 2.4.b). In this case, statistically significant differences in $\log_{10}$ inactivation were observed for both MNV-1 and FCV at each of the three concentrations tested (5, 75, and 250 ppm). More specifically, at the highest concentration tested (250 ppm), 3.9 and 2.5 $\log_{10}$ inactivation in RNA copy number was observed for MNV-1 and FCV, respectively.

Quaternary ammonium compound B was ineffective at inactivating all four viruses, with <0.5 $\log_{10}$ reductions at all concentrations tested regardless of assay type, i.e., RT-qPCR or infectivity (Figure 2.5.a. and 2.5.b)

A separate statistical analysis was undertaken to compare disinfection efficacy for ethanol and hypochlorite as a function of concentration, as evaluated by RT-qPCR. At 50% ethanol, all viruses showed relative resistance ($\leq$0.6 $\log_{10}$ reduction), while at 70% and 90%, MNV-1 was significantly more susceptible than the other three viruses (Figure 2.6.a). The virus-specific differences were much more pronounced for hypochlorite treatment (Figure 2.6.b). Specifically, while all four viruses remained relatively unaffected at low concentrations ($\leq$75 ppm), the animal surrogates were much more sensitive to higher hypochlorite concentrations ($\geq$250 ppm), as evaluated by RT-PCR.

Statistical analysis was also done to compare mammalian cell culture infectivity data to RT-qPCR for the two animal surrogate viruses treated with ethanol and hypochlorite; the quaternary ammonium compound was excluded from this analysis due to its poor overall efficacy. For MNV-1, there were statistically significant differences between the two assay types at ethanol concentrations of 70% and 90% (Figure 2.7.a), but this is of limited practical significance as these values exceeded assay limits of detection. At 75 ppm hypochlorite, there was a highly significant difference between RT-qPCR and cell culture infectivity assay results for MNV-1, less for the 250 ppm treatment. At higher disinfectant concentrations, virus titers fell to below detection limits for both assays (Figure 2.5b). On the whole, there was relatively good correlation between RT-

qPCR and infectivity assay for FCV for all disinfectants at all concentrations, with no statistically significant differences observed at any concentration (Figure 2.8). At 500 ppm hypochlorite, comparisons between infectivity assay and RT-qPCR were not possible due to residual cytotoxicity.

2.5 DISCUSSION

The purpose of this study was to compare the efficacy of three commonly used disinfectants (ethanol, hypochlorite, and quaternary ammonium) at multiple concentrations, against HuNoV and two cultivable surrogates (FCV and MNV-1), using a combination of cell culture infectivity assay and RT-qPCR. The experimental design and subsequent statistical analysis allowed us to investigate three major relationships: (i) the effect of disinfectant concentration of virus inactivation; (ii) the relationship between disinfectant efficacy as evaluated by RT-qPCR and infectivity assay; and (iii) the difference between the efficacies of the disinfectants against HuNoV vs. the cultivable surrogate viruses.

Ethanol is commonly used as an active component in hand sanitizers as it is relatively mild and easily combined with other agents to improve its efficacy and provide products with consumer appeal. Comparatively speaking, the HuNoV and FCV were more resistant to ethanol than was MNV-1. In fact, ethanol, at any concentration tested (50, 70, and 90%) failed to inactivate the former viruses to any substantial degree, while MNV-1 was inactivated approximately 3.5 $\log_{10}$ at the higher ethanol concentrations (70

and 90%). Many studies have sought to characterize the efficacy of ethanol against FCV, with widely variable results. A few have reported a high degree of efficacy. For example, Macinga et al (2008) found that, by infectivity assay, 70% ethanol was able to inactivate FCV $\geq$4.75 $\log_{10}$ after a 30 second exposure (27). Gehrke et al (2004), using both suspension and fingerpad methods, showed that 70% ethanol treatment resulted in approximately 4 $\log_{10}$ reductions in FCV after 1-3 minutes of contact (13).

On the other hand, the vast majority of studies show that ethanol is relatively ineffective against FCV. For example, using the fingerpad methods and infectivity assay, Lages et al (2008), found that FCV was not significantly inactivated ($<1 \log_{10}$) at ethanol concentrations of 62% or 99.5% with contact times ranging from 30 sec to 2 min (25). The findings of Park et al (2010) were similar, with FCV being inactivated $\leq 0.3 \log_{10}$ after treatments with 50, 70 and 90% ethanol after a 5 minute contact time, as evaluated by RT-qPCR. Others have reported more intermediate results, such as those of Doultree et al (1999) and Whitehead and McCue (2010), who both reported approximately 1.3 $\log_{10}$ reduction in FCV infectivity after a 1 minute exposure to 60-75% ethanol using suspension and surface test formats (9) (46). Park et al (2010) also reported that treatment with 50 and 70% ethanol resulted in a 2.2 and 2.6 $\log_{10}$ reduction in FCV infectivity after a 5 minute contact time (33). Even with extended contact times, studies have failed to demonstrate significant inactivation of FCV by ethanol. Malik and Goyal, (2006) demonstrated a 85-99.5% reduction in infectious virus inoculated onto stainless steel for contact times varying from 1-10 minutes, with minimal concentration effect (29).

Duizer et al (2004) found that this virus was inactivated <2 $\log_{10}$ after an 8 minute exposure to 70% ethanol, and a 3 $\log_{10}$ reduction was observed after a 30 minute exposure as determined by infectivity assay(10).

Consistent with other studies, we did observe that MNV-1 was quite susceptible to ethanol. For instance, in suspension tests, Park et al (2010) observed that ethanol concentrations > 70% resulted in > 3 $\log_{10}$ reduction in virus titer by as evaluated by both infectivity assay and RT-qPCR (33). Likewise, Mugulski et al.(2009) demonstrated concentration-dependent inactivation of MNV-1, i.e. a 2 $\log_{10}$ reduction at 45% ethanol and a >6 $\log_{10}$ reduction at $\geq$ 55% ethanol after a 5 minute contact period based on the quantitative carrier test and infectivity assay (31). Macinga et al (2008) observed a $\geq$3.7 $\log_{10}$ reduction of MNV-1 in suspension assay, and a 2.5 $\log_{10}$ inactivation in the finger pad assay, both after treatment for 30 seconds with an ethanol-based hand sanitizer (27). Belliot et al (2008) found that concentrations $\geq$ 60% ethanol resulted in a 4 $\log_{10}$ reduction at contact times ranging from 30 seconds to 3 minutes, with results consistent for both infectivity and RT-qPCR assays (2).

A relatively few studies have investigated the efficacy of ethanol against HuNoV. Duizer et al (2004) found that ethanol failed to completely inactivate HuNoV (as evaluated by RT-qPCR), although with 70% ethanol, virus titer did show a decrease of <2 $\log_{10}$ after 8 minutes, a trend similar to that observed for the surrogate viruses (FCV, CaCV) (10). Similar findings were reported by Liu et al (2010) who showed a no more than 0.5 $\log_{10}$ reduction in HuNoV RNA after a 30 second contact time, regardless of

ethanol concentration (26). Park et al (2010) also observed minimal inactivation (<1 log_{10}) of HuNoV after treatment for 5 minutes at various ethanol concentrations (33).

Quaternary ammonium compounds (QUATS) are another commonly used commercial disinfectant category. Many studies support the relative lack of efficacy of these disinfectants against nonenveloped RNA viruses (15), which is intuitive as these are lipophilic compounds and human enteric viruses lack a lipid envelope (1, 9, 19). Indeed, QUAT B was ineffective in inactivating any of the viruses tested in our study, as evaluated by RT-qPCR or infectivity assay at low QUAT concentrations; at high concentrations, the QUAT compound was cytotoxic. This agrees with previous studies, including Doultree et al (1999) and Whitehead and McCue (2010), who found that QUAT compounds have little effect on FCV (9, 46).

Sodium hypochlorite is the active compound in bleach and behaves as a very strong oxidizing agent with its degree of activity dependent upon both concentration and contact time (46). As is the case for ethanol, many studies have been done to evaluate the antiviral efficacy of hypochlorite, with mixed results. Consistent with our work, Whitehead and McCue (2010) also found that 100 ppm free chlorine was able to inactivate FCV by only 2.3 log_{10}, while 1000 ppm resulted in a > 4.2 log_{10} reduction as determined by infectivity assay (46). However, many others report that very high chlorine concentrations were necessary to inactivate FCV. For example, Doultree et al (1999) found that treatment with 5000 ppm resulted in complete inactivation of FCV (>5 log_{10}), while <1000 ppm provided <2.8 log_{10} reduction after a 1 minute exposure (9).

Poschetto et al (2007) reported that $\geq$ 4000 ppm available chlorine was necessary to reduce the infectivity of FCV >3 $\log_{10}$ in suspension test with a 15 minute exposure period (36), while Duizer et al. (2004) used 3,000 ppm to obtain > 5 $\log_{10}$ inactivation after 10-30 minute contact (10). On food contact surfaces and foods (strawberry and lettuce), Gulati et al (2001) found that <800 ppm free chlorine had little effect on FCV infectivity, while 5000 ppm produced a 3.4 $\log_{10}$ reduction (17).

Fewer studies have been done with MNV-1 or HuNoV. Belliot et al. (2008) observed >4 $\log_{10}$ reduction in MNV-1 titer after treatment with 5000 ppm free chlorine for 30 sec (2); similarly, Girard et al. (2010) observed that HuNoV titer was reduced after exposure to 570 ppm free chlorine by 2 $\log_{10}$ (5 min contact time) and ≥3 $\log_{10}$ (10 min contact time). MNV-1 was completely inactivated by 570 ppm free chlorine at both 5 and 10 minutes, as evaluated by infectivity assay, with a 4 $\log_{10}$ reduction documented using RT-qPCR applied after an enzymatic pretreatments (15). In recent work, Liu et al. (2010) reported that, using both suspension and fingerpad methods, treatment with free chlorine at concentrations $\geq$160 ppm for 30 seconds resulted in a 5 $\log_{10}$ reduction in Norwalk virus by RT-qPCR (26). Shin and Sobsey (2008) reported that Norwalk virus was highly sensitive to chlorine, with a 3 $\log_{10}$ reduction when exposed to 1 ppm free chlorine for 10 minutes, or 5 ppm for <1 minute, in the suspension assay (42).

Most studies undertaken to evaluate the efficacy of disinfecting agents against HuNoV have been applied either solely to human strains using RT-PCR, or else to surrogates whose survival was assessed exclusively using infectivity assay. Very few

studies have included RT-qPCR with infectivity assay (for surrogates) or have compared surrogates with HuNoV strains. Compared to the body of prior evidence, our work demonstrates some unique findings; specifically that HuNoV may well be more resistant to chlorine than are the animal surrogates. More specifically, efficacy against FCV and MNV-1 was observed at chlorine concentrations >250 ppm, but for the human strains, concentrations of 500-≥1000 ppm were needed before significant inactivation was observed. This has important implications when making decisions about strategies to inactivate HuNoV based exclusively on the data from surrogates.

Even though RT-qPCR tended to underestimate inactivation relative to infectivity data, the inactivation trends (i.e., reduction in virus titer) were similar when comparing the two assay methods. The only exception to this trend was for MNV-1 with hypochlorite, in which case there was a large discrepancy between log reduction by infectivity assay and RT-qPCR at a concentration of 75 ppm. Other investigators have observed similar trends for MNV-1 (15, 22) and FCV (10).

Disinfectants have different modes of action. Ethanol appears to work at the capsid level; hypochlorite may work on both capid protein and viral RNA (26, 30, 32). Consequently, one might anticipate a better correlation between data on virus inactivation obtained using RT-qPCR vs. infectivity assay for chlorine as compared to ethanol. Indeed, when the surrogate viruses were treated with higher concentrations of hypochlorite (≥ 250 ppm), virus titers were below detection limits by both infectivity assay and RT-qPCR. However, for ethanol there was also a relatively good correlation between inactivation

data obtained using the two assays. It would be appropriate to further explore this relationship in future work, by preceding RT-qPCR with sample pretreatments that are designed to inactivate free RNA and/or damaged capsids. Candidate methods might include enzymatic pretreatments (proteinase K and RNase) (38) and nucleic acid intercalating agents (40).

Antibody-bound magnetic beads have been widely used to aid in the concentration of enteric viruses from complex matrices such as food, environmental, or clinical (fecal) samples in preparation for detection by RT-PCR (20, 23, 34, 40).It has been argued that, in addition to facilitating virus concentration with removal of matrix-associated inhibitors, the use of IMS may facilitate the recovery of infectious virus to the exclusion of non-infectious virus(14). Although not a direct measure of virus infectivity, antibody capture does circumvent the problem of detection of naked viral RNA, which can result from capsid disruption occurring as a consequence of virus inactivation (14, 20).

It is well recognized that chlorine compounds are readily inactivated by organic matter and that fecal matter may actually protect viruses by rapidly contributing to depletion of free chlorine. Viruses may also be shielded from active disinfection products by attachment to organic matter (4). Furthermore, HuNoV have a tendency to aggregate in naturally contaminated samples (feces) which may also contribute to increased disinfection resistance (26, 43). For example, Urakami et al (2007) found that the presence of host cellular debris affected the efficacy of hypochlorite in inactivation of

FCV(45). Others, however, have found that elevated soil load had limited effect on disinfection efficacy (27, 28). Curiously, we observed little difference in inactivation efficacy, by either cell culture infectivity assay or RT-qPCR, when repeating the experiments with MNV-1 suspended in artificial feces (data not shown). Others have observed the same with HuNoV surrogates and have suggested that these clay-based matrices may not be truly representative of human feces (3, 27, 28). Further studies in which the cultivable surrogates are suspended in human feces and subjected to disinfection are in progress in our laboratory.

Our study has some limitations. For example, data collection was restricted to a 30 second contact time and hence did not evaluate the combined effects of sanitizer concentration and time. Also, the suspension assay method was used, which may produce results not directly comparable to surface disinfection. Another limitation includes the differences in the virus suspension matrix for HuNoV (20% feces suspended in PBS) and MNV-1 and FCV (cell culture lysate).

To the best of our knowledge, this is the first comprehensive comparative study in which the efficacy of three commonly used disinfectants was compared at multiple concentrations and with both HuNoV and cultivable animal surrogates, with inclusion of both infectivity assay and RT-qPCR. Our major findings can be summarized as follows: (i) MNV-1 was the only virus susceptible to high concentrations of ethanol; (ii) when comparing RT-qPCR results by virus types, the HuNoV appear to be more resistant to both hypochlorite and ethanol than MNV-1 and FCV; and (iii) although RT-qPCR had a

tendency to underestimate degree of virus inactivation, there was still some correlation between results obtained by infectivity assay and molecular amplification for the cultivable surrogates.

The results of this study suggest that ethanol and quaternary ammonium are ineffective at inactivation of HuNoV, as is hypochlorite at concentrations routinely used in the farm-to-fork chain. The poor efficacy of these disinfectants has important implications for controlling virus transmission. There is a clear need to develop disinfectants with improved antiviral efficacy, and perhaps, better methods to promote user compliance with hand and surface disinfection protocols, particularly in the food service sector. This study also highlights the differences in behavior of HuNoV and the cultivable surrogates with respect to disinfection efficacy, and the continuing need for reliable methods to predict HuNoV infectivity in the absence of an *in vitro* cultivation method.

Table 2.1 Primers and Probes for detecting viruses by RT-qPCR

Norovirus Strain	Oligonucleotide (primer)	Sequence	Nucleotide position	Reference
Human norovirus genogroup II.2 and genogroup II.4	JJV2F	5' CAAGAGTCAATGTTTAGGTGGATGAG 3'	5003-5028	Jothikumar, et al. (2005)
	COG2R	5' -GACGCCATCTTCATTCACA-3'	5100-5080	
	RING2P	5' 56-FAMTGGGAGGGCGATCGCAATCT-3BHQ 1-3'	5048-5067	
Murine norovirus	G54763F	5'TGATCGTGCCAGCATCGA3'	4763-4781	Vinje, unpublished
	G54863R	5'GTTGGGAGGGTCTCTGAGCAT 3'	4863-4884	
	G54808P	5' 56FAMCTACCCACCAGAACCCCTTTGAGACTC3BHQ 13'	4808-4835	
Feline Calicivirus	FCV F	5' GCCAATCAGCATGTGGTAACC 3'	2429-2449	Rawsthorne this work
	FCV R	5' GCACATCATATGCGGCTCTG 3'	2540-2521	
	FCV P	5'- /56-FAM/CCCAGGCCAAATCAAACACCGAATTAA /3BHQ_1/ -3'	2451-2477	

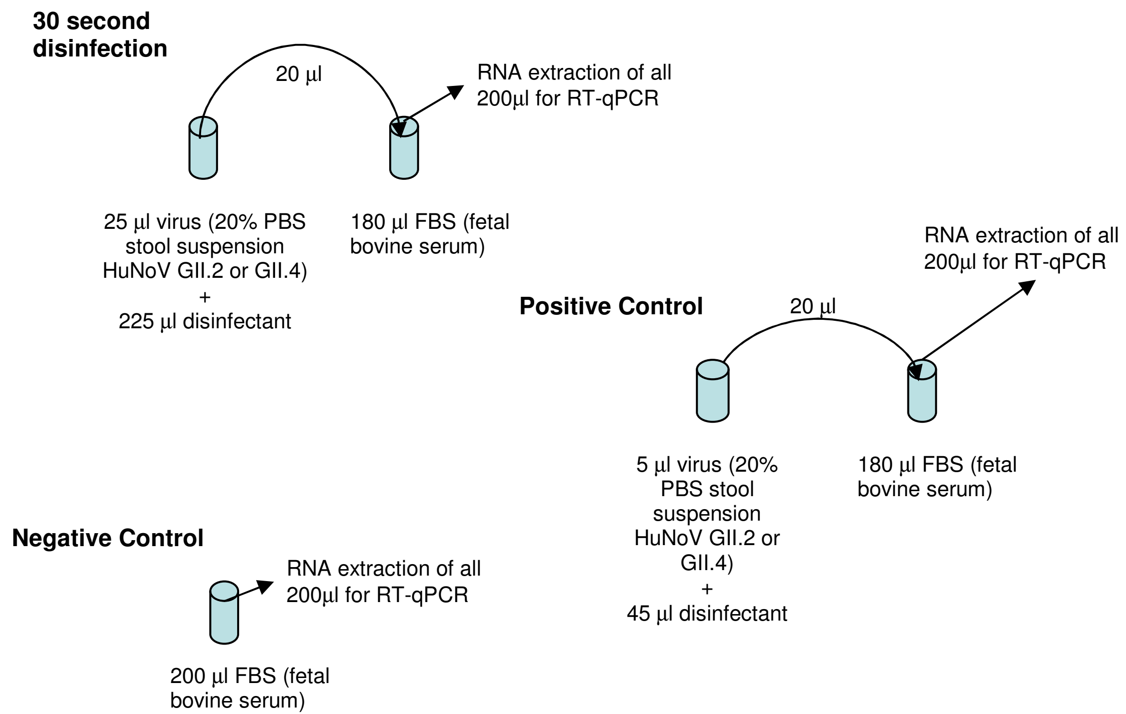


Figure 2.1 Schematic for HuNoV GII.2 and GII.4 Disinfections

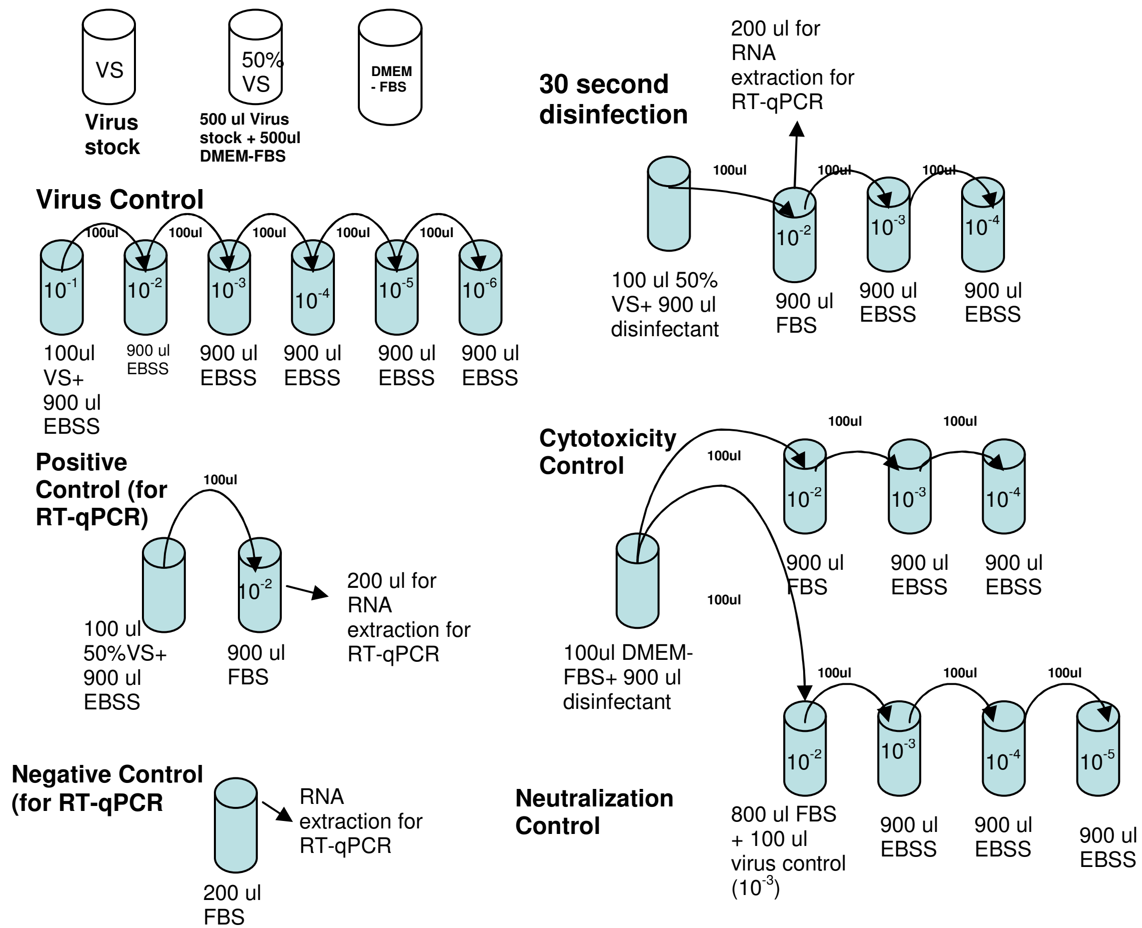
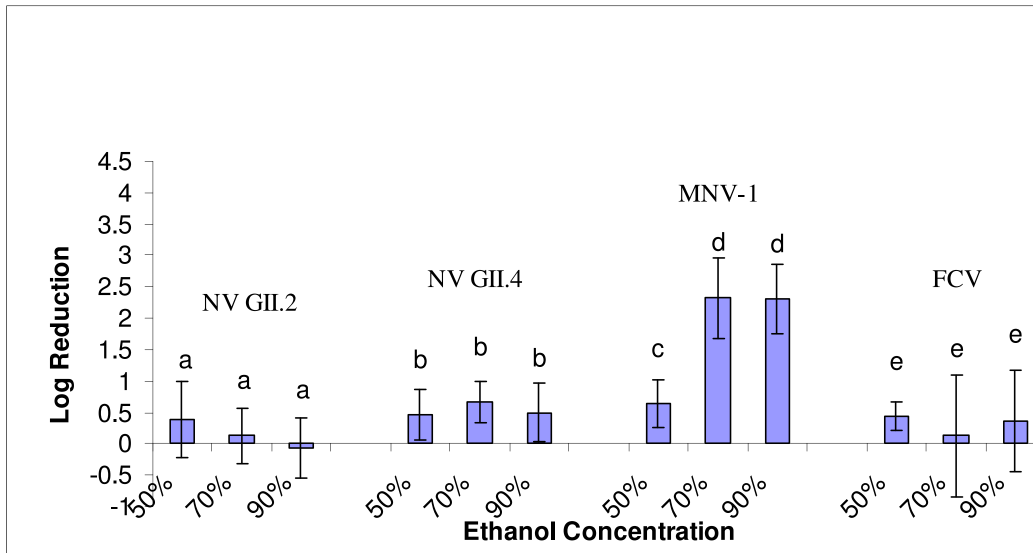


Figure 2.2 Schematic for MNV-1 and FCV Disinfection.

For infectivity assays, the following were plated: virus controls (10^{-4}-10^{-6} dilutions) in duplicate; 30 second disinfection (10^{-2}-10^{-4}) in triplicate; cytotoxicity control (10^{-2}-10^{-4}) in triplicate; neutralization control (10^{-2}-10^{-5}) in triplicate; and a cell culture control of just 200 ul EBSS was plated in triplicate.

A



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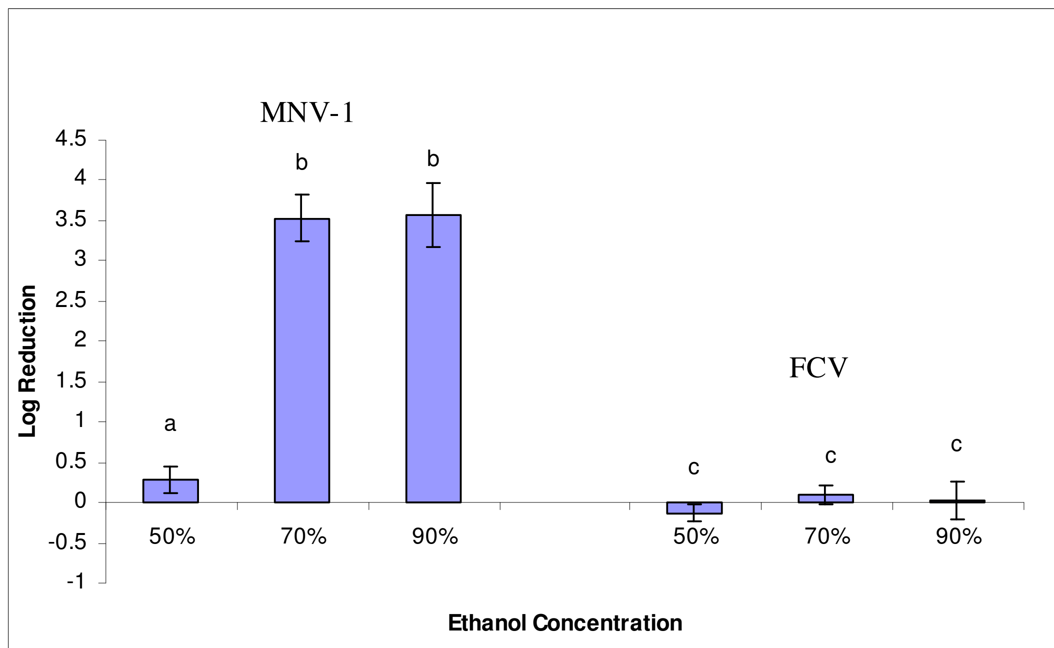
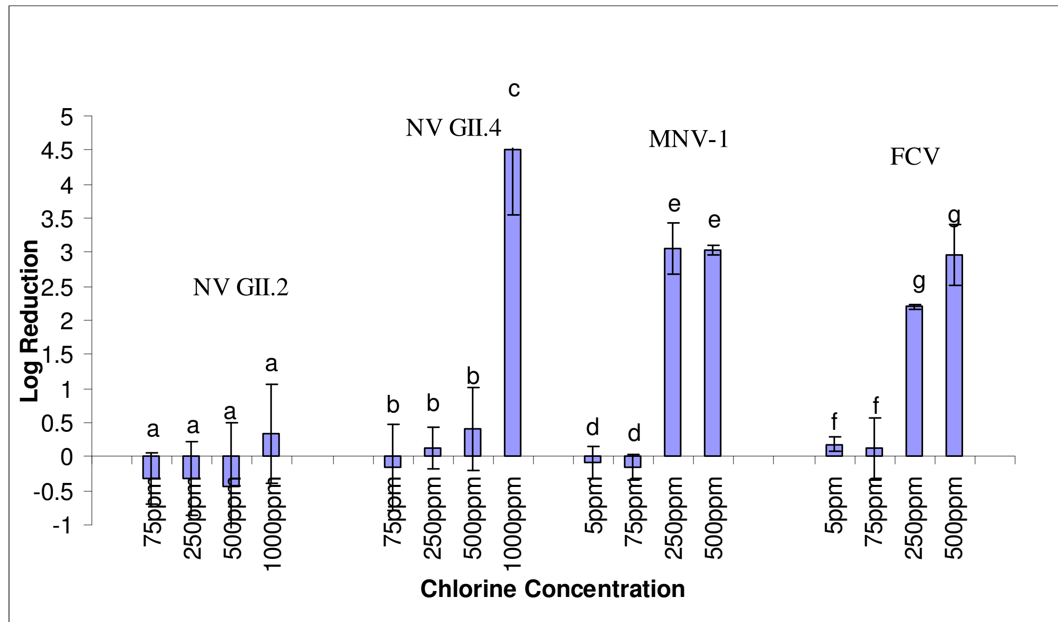


Figure 2.3. Comparison of virus inactivation efficacy as determined by (a) RT-qPCR and (b) mammalian cell culture infectivity assay after treatment with ethanol (suspension test) at different concentrations for HuNoV GII.2, HuNoV GII.4, MNV-1 and FCV.

A



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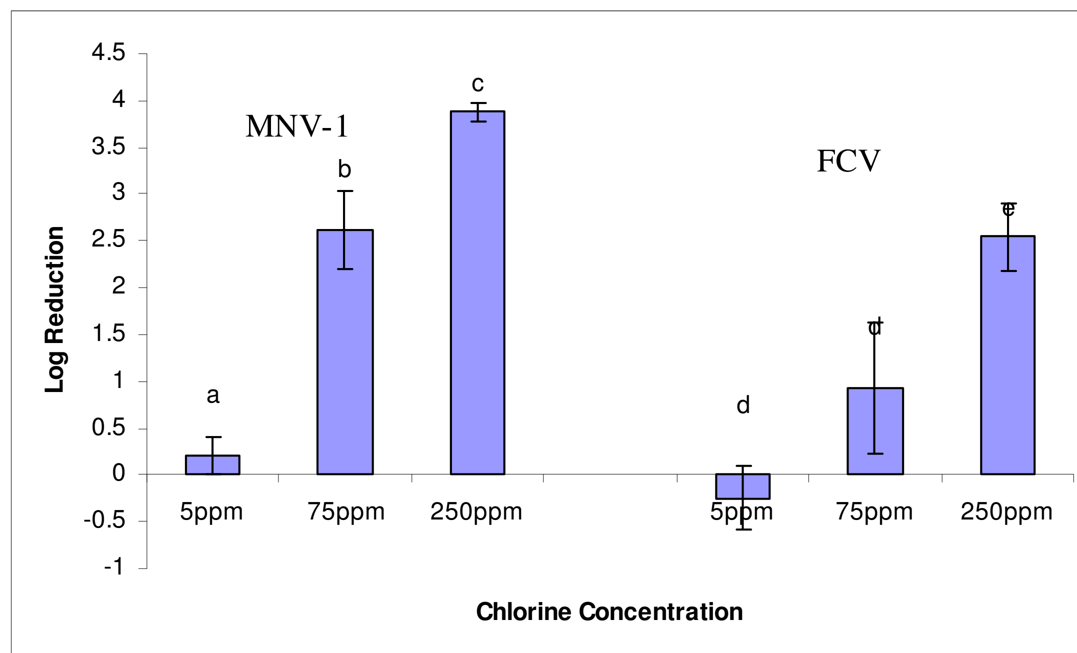
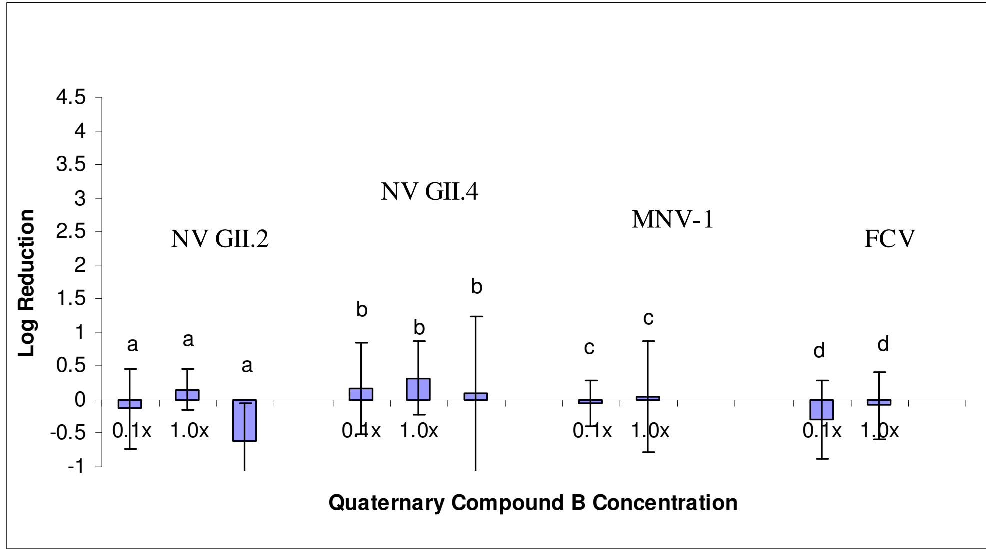


Figure 2.4. Comparison of virus inactivation efficacy as determined by (a) RT-qPCR and (b) mammalian cell culture infectivity assay after treatment with sodium hypochlorite (suspension test) at different concentrations for HuNoV GII.2, HuNoV GII.4, MNV-1 and FCV.

A



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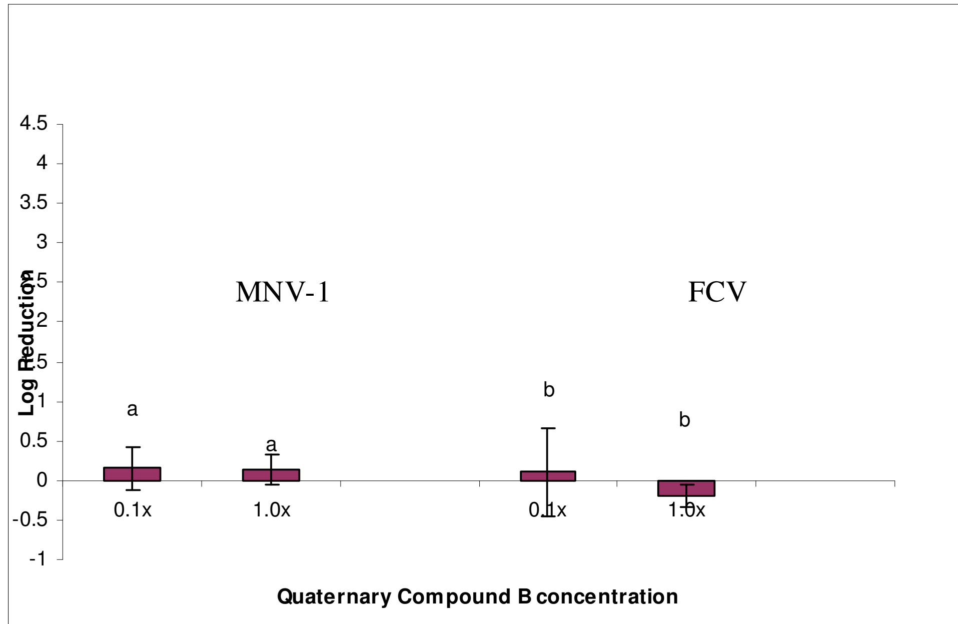
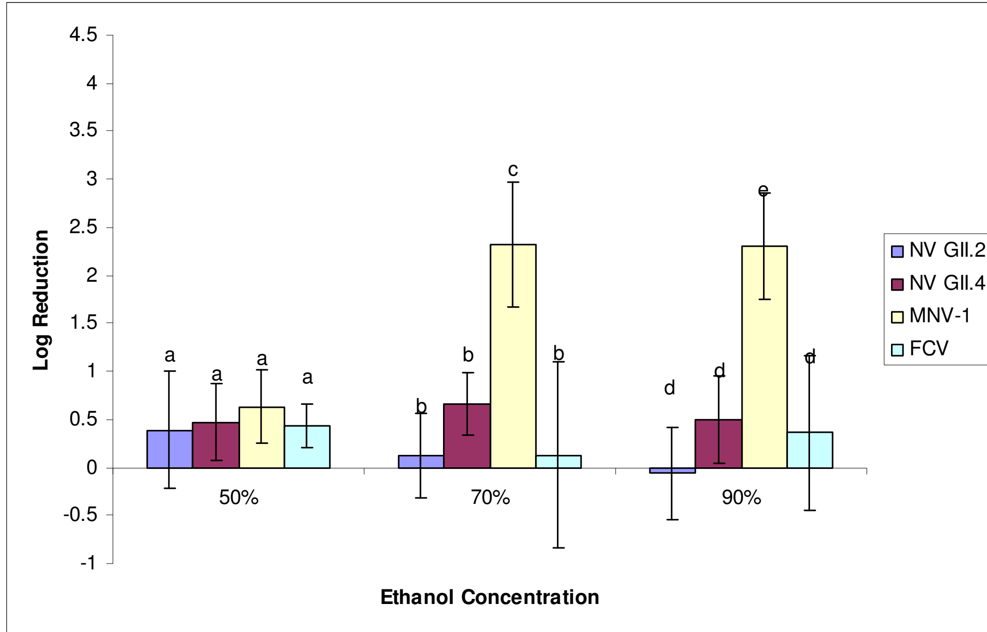


Figure 2.5. Comparison of virus inactivation efficacy as determined by (a) RT-qPCR and (b) mammalian cell culture infectivity assay after treatment with quaternary ammonium compound B (suspension test) at different concentrations for HuNoV GII.2, HuNoV GII.4, MNV-1 and FCV.

Figure 2.6. Comparison of inactivation of HuNoV GII.2, GII.4, MNV-1 and FCV by RT-qPCR as a function of disinfectant concentration for (a) ethanol; (b) hypochlorite; and (c) quaternary ammonium compound B.

A



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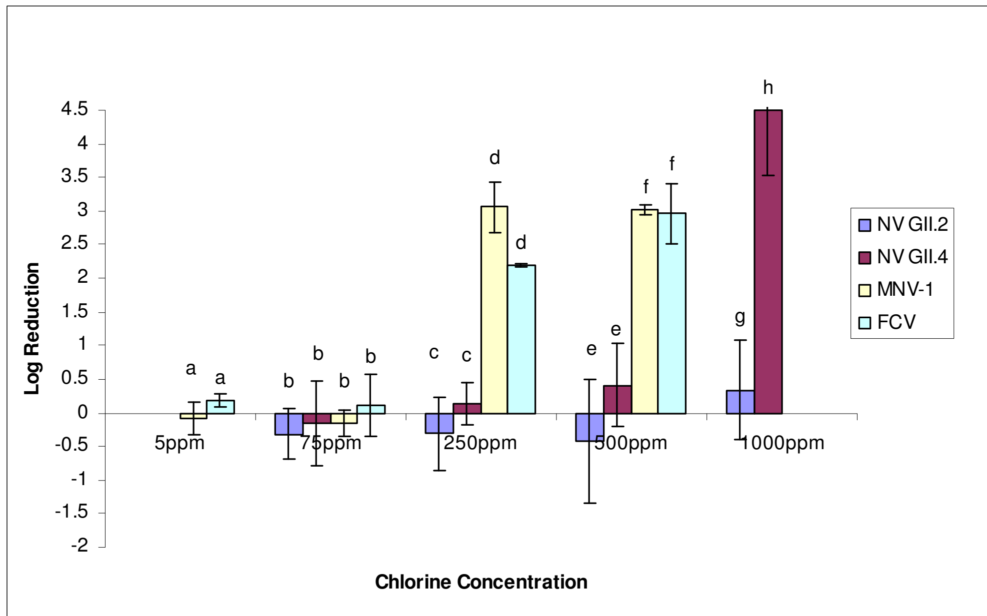

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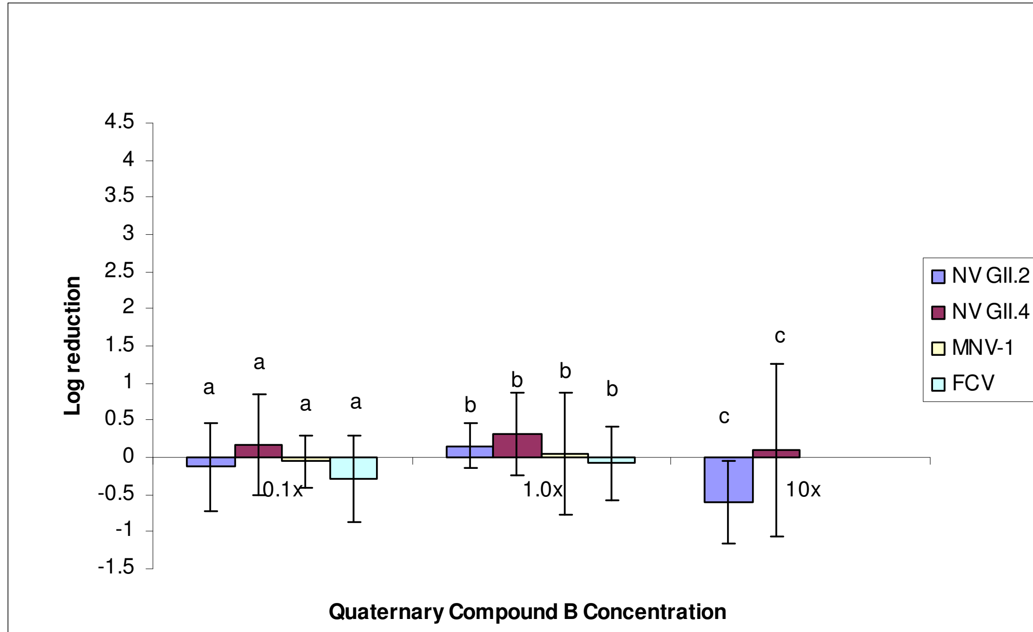

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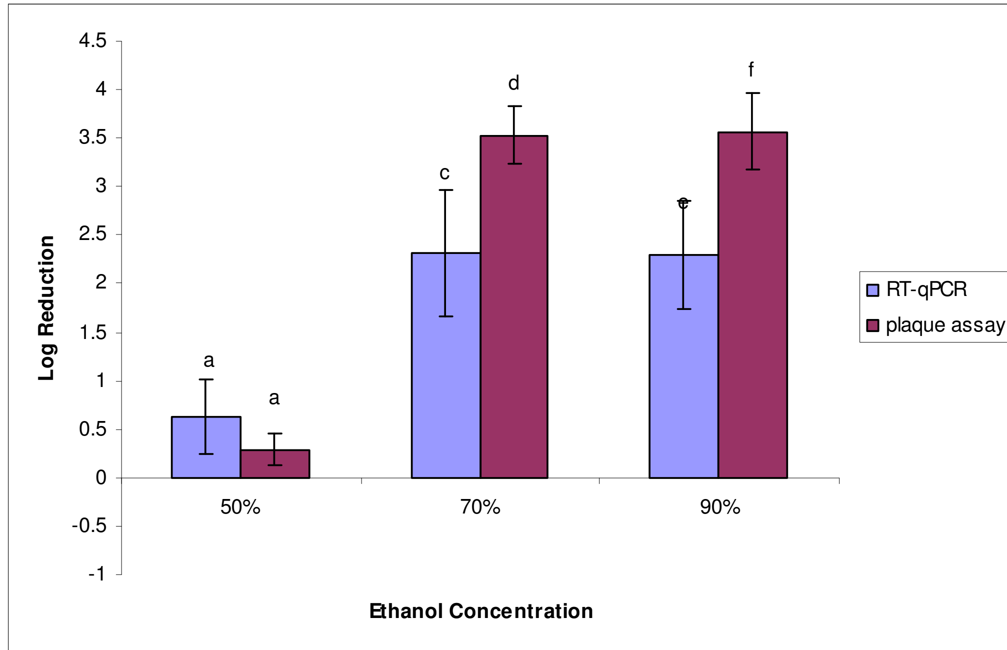

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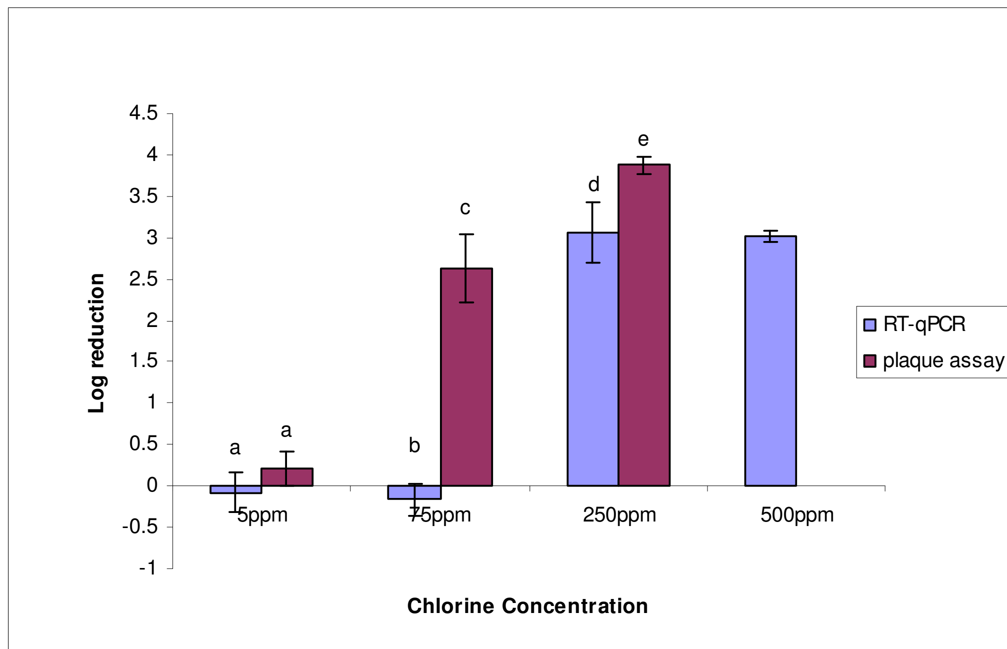
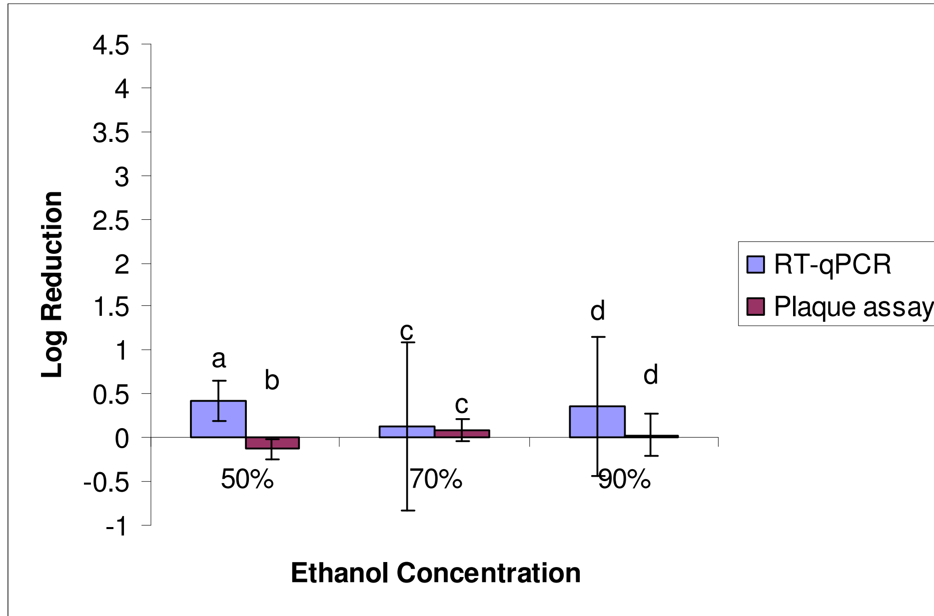


Figure 2.7. Comparison RT-qPCR and mammalian cell culture infectivity assay data on the inactivation efficiency of various concentrations of (a) ethanol; (b) sodium hypochlorite as applied to MNV-1.

A



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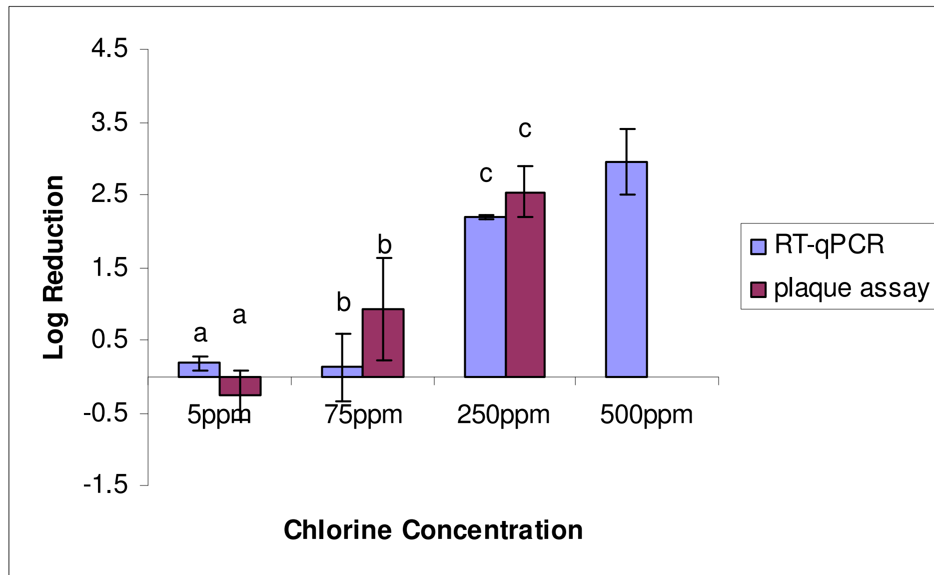
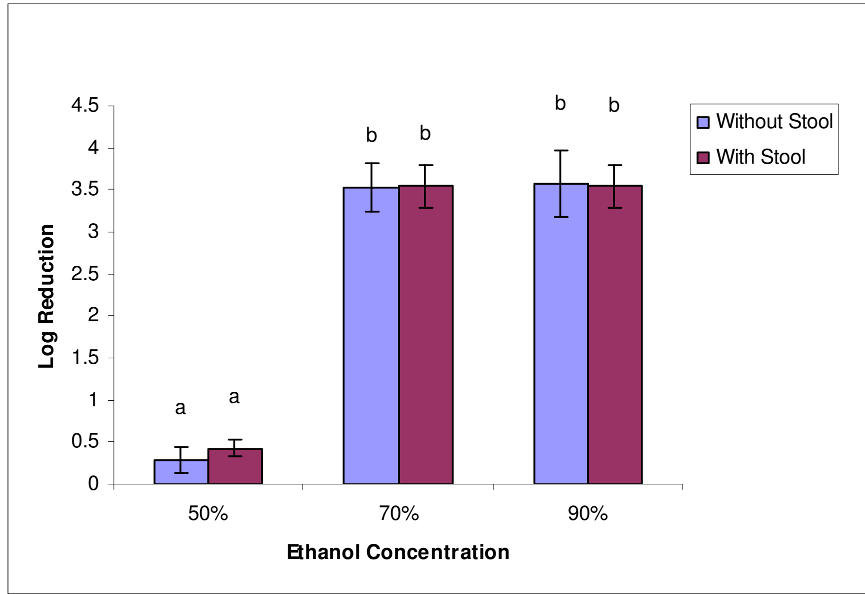


Figure 2.8. Comparison RT-qPCR and mammalian cell culture infectivity assay data on the inactivation efficiency of various concentrations of (a) ethanol and (b) sodium hypochlorite.

A



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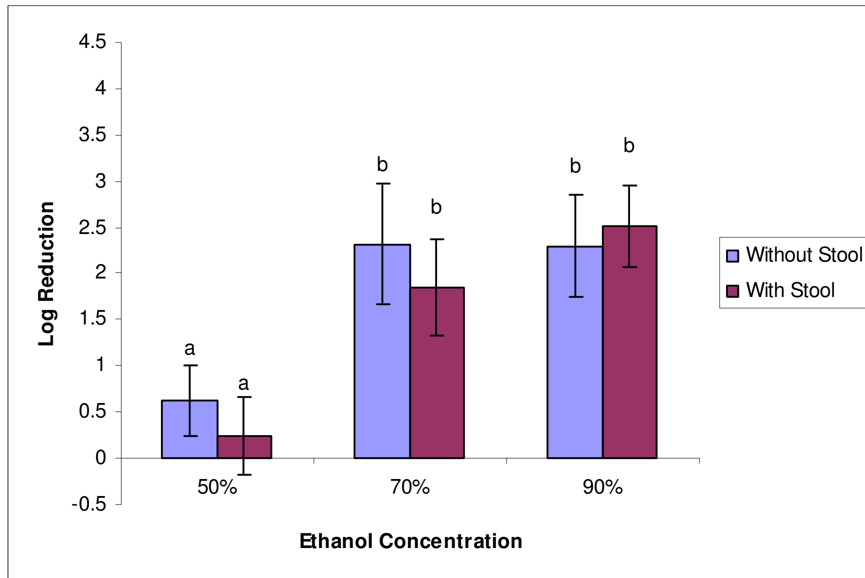


Figure 2.9 Comparison MNV-1 with stool and without stool on the inactivation efficiency of various concentrations ethanol using (a) infectivity assay and (b) RT-qPCR.

2.6 REFERENCES

1. **Abad, F. X., R. M. Pinto, and A. Bosch.** 1997. Disinfection of human enteric viruses on fomites. FEMS Microbiol Lett **156:**107-11.

2. **Belliot, G., A. Lavaux, D. Souihel, D. Agnello, and P. Pothier.** 2008. Use of murine norovirus as a surrogate to evaluate resistance of human norovirus to disinfectants. Appl Environ Microbiol **74:**3315-8.

3. **Cannon, J. L., E. Papafragkou, G. W. Park, J. Osborne, L. A. Jaykus, and J. Vinje.** 2006. Surrogates for the study of norovirus stability and inactivation in the environment: aA comparison of murine norovirus and feline calicivirus. J Food Prot **69:**2761-5.

4. **Chaidez, C., M. Moreno, W. Rubio, M. Angulo, and B. Valdez.** 2003. Comparison of the disinfection efficacy of chlorine-based products for inactivation of viral indicators and pathogenic bacteria in produce wash water. Int J Environ Health Res **13:**295-302.

5. **Cheesbrough, J. S., L. Barkess-Jones, and D. W. Brown.** 1997. Possible prolonged environmental survival of small round structured viruses. J Hosp Infect **35:**325-6.

6. **Cheesbrough, J. S., J. Green, C. I. Gallimore, P. A. Wright, and D. W. Brown.** 2000. Widespread environmental contamination with Norwalk-like viruses (NLV) detected in a prolonged hotel outbreak of gastroenteritis. Epidemiol Infect **125:**93-8.

7. **Choi, S., and S. C. Jiang.** 2005. Real-time PCR quantification of human adenoviruses in urban rivers indicates genome prevalence but low infectivity. Appl Environ Microbiol **71:**7426-33.

8. **D'Souza, D. H., A. Sair, K. Williams, E. Papafragkou, J. Jean, C. Moore, and L. Jaykus.** 2006. Persistence of caliciviruses on environmental surfaces and their transfer to food. Int J Food Microbiol **108:**84-91.

9. **Doultree, J. C., J. D. Druce, C. J. Birch, D. S. Bowden, and J. A. Marshall.** 1999. Inactivation of feline calicivirus, a Norwalk virus surrogate. J Hosp Infect **41:**51-7.

10. **Duizer, E., P. Bijkerk, B. Rockx, A. De Groot, F. Twisk, and M. Koopmans.** 2004. Inactivation of caliciviruses. Appl Environ Microbiol **70:**4538-43.

11. **Espinosa, A. C., M. Mazari-Hiriart, R. Espinosa, L. Maruri-Avidal, E. Mendez, and C. F. Arias.** 2008. Infectivity and genome persistence of rotavirus and astrovirus in groundwater and surface water. Water Res **42:**2618-28.

12. **FDA.** 1994. Topical antimicrobial products for over-the-counter use; tentative final monograph for healthcare antiseptic drug products. Federal Register **59:**31221-2.

13. **Gehrke, C., J. Steinmann, and P. Goroncy-Bermes.** 2004. Inactivation of feline calicivirus, a surrogate of norovirus (formerly Norwalk-like viruses), by different types of alcohol in vitro and in vivo. J Hosp Infect **56:**49-55.

14. **Gilpatrick, S. G., K. J. Schwab, M. K. Estes, and R. L. Atmar.** 2000. Development of an immunomagnetic capture reverse transcription-PCR assay for the detection of Norwalk virus. J Virol Methods **90:**69-78.

15. **Girard, M., S. Ngazoa, K. Mattison, and J. Jean.** 2010. Attachment of noroviruses to stainless steel and their inactivation, using household disinfectants. J Food Prot **73:**400-4.

16. **Green, J., K. Henshilwood, C. I. Gallimore, D. W. Brown, and D. N. Lees.** 1998. A nested reverse transcriptase PCR assay for detection of small round-structured viruses in environmentally contaminated molluscan shellfish. Appl Environ Microbiol **64:**858-63.

17. **Gulati, B. R., P. B. Allwood, C. W. Hedberg, and S. M. Goyal.** 2001. Efficacy of commonly used disinfectants for the inactivation of calicivirus on strawberry, lettuce, and a food-contact surface. J Food Prot **64:**1430-4.

18. **Huss, H. H.** 2003, posting date. Assessment and management of sea food safety and quality. Food Agriculture Organization (FAO). [Online.]

19. **Jean, J., J. F. Vachon, O. Moroni, A. Darveau, I. Kukavica-Ibrulj, and I. Fliss.** 2003. Effectiveness of commercial disinfectants for inactivating hepatitis A virus on agri-food surfaces. J Food Prot **66:**115-9.

20. **Jothikumar, N., D. O. Cliver, and T. W. Mariam.** 1998. Immunomagnetic capture PCR for rapid concentration and detection of hepatitis A virus from environmental samples. Appl Environ Microbiol **64:**504-8.

21. **Karst, S. M., C. E. Wobus, M. Lay, J. Davidson, and H. W. t. Virgin.** 2003. STAT1-dependent innate immunity to a Norwalk-like virus. Science **299:**1575-8.

22. **Kitajima, M., Y. Tohya, K. Matsubara, E. Haramoto, E. Utagawa, and H. Katayama.** 2010. Chlorine inactivation of human norovirus, murine norovirus and poliovirus in drinking water. Lett Appl Microbiol **51:**119-21.

23. **Kobayashi, S., K. Natori, N. Takeda, and K. Sakae.** 2004. Immunomagnetic capture rt-PCR for detection of norovirus from foods implicated in a foodborne outbreak. Microbiol Immunol **48:**201-4.

24. **Koopmans, M., and E. Duizer.** 2004. Foodborne viruses: an emerging problem. Int J Food Microbiol **90:**23-41.

25. **Lages, S. L., M. A. Ramakrishnan, and S. M. Goyal.** 2008. In-vivo efficacy of hand sanitisers against feline calicivirus: a surrogate for norovirus. J Hosp Infect **68:**159-63.

26. **Liu, P., Y. Yuen, H. M. Hsiao, L. A. Jaykus, and C. Moe.** 2010. Effectiveness of liquid soap and hand sanitizer against Norwalk virus on contaminated hands. Appl Environ Microbiol **76:**394-9.

27. **Macinga, D. R., S. A. Sattar, L. A. Jaykus, and J. W. Arbogast.** 2008. Improved inactivation of nonenveloped enteric viruses and their surrogates by a novel alcohol-based hand sanitizer. Appl Environ Microbiol **74:**5047-52.

28. **Magulski, T., D. Paulmann, B. Bischoff, B. Becker, E. Steinmann, J. Steinmann, P. Goroncy-Bermes, and J. Steinmann.** 2009. Inactivation of murine norovirus by chemical biocides on stainless steel. BMC Infect Dis **9:**107.

29. **Malik, Y. S., and S. M. Goyal.** 2006. Virucidal efficacy of sodium bicarbonate on a food contact surface against feline calicivirus, a norovirus surrogate. Int J Food Microbiol **109:**160-3.

30. **McDonnell, G., and A. D. Russell.** 1999. Antiseptics and disinfectants: activity, action, and resistance. Clin Microbiol Rev **12:**147-79.

31. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. Emerg Infect Dis **5:**607-25.

32. **Nuanualsuwan, S., and D. O. Cliver.** 2003. Infectivity of RNA from inactivated poliovirus. Appl Environ Microbiol **69:**1629-32.

33. **Park, G. W., Barclay, L., Macinga, D., Charbonneau, D., Pettigrew, C.A., Vinje, J.** 2010. Comparative Efficacy of Seven Hand Sanitizers against Murine Norovirus, Feline Calicivirus and GII.4 Norovirus. Journal of Food Protection **In Press**.

34. **Park, Y., Y. H. Cho, Y. Jee, and G. Ko.** 2008. Immunomagnetic separation combined with real-time reverse transcriptase PCR assays for detection of norovirus in contaminated food. Appl Environ Microbiol **74:**4226-30.

35. **Patel, M. M., A. J. Hall, J. Vinje, and U. D. Parashar.** 2009. Noroviruses: a comprehensive review. J Clin Virol **44:**1-8.

36. **Poschetto, L. F., A. Ike, T. Papp, U. Mohn, R. Bohm, and R. E. Marschang.** 2007. Comparison of the sensitivities of noroviruses and feline calicivirus to chemical disinfection under field-like conditions. Appl Environ Microbiol **73:**5494-500.

37. **Rockx, B., M. De Wit, H. Vennema, J. Vinje, E. De Bruin, Y. Van Duynhoven, and M. Koopmans.** 2002. Natural history of human calicivirus infection: a prospective cohort study. Clin Infect Dis **35:**246-53.

38. **Scallan, E., P. M. Griffin, F. J. Angulo, R. V. Tauxe, and R. M. Hoekstra.** Foodborne illness acquired in the United States--unspecified agents. Emerg Infect Dis **17:**16-22.

39. **Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin.** Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis **17:**7-15.

40. **Schwab, K. J., R. De Leon, and M. D. Sobsey.** 1996. Immunoaffinity concentration and purification of waterborne enteric viruses for detection by reverse transcriptase PCR. Appl Environ Microbiol **62:**2086-94.

41. **Shieh, Y. C., C. I. Wong, J. A. Krantz, and F. C. Hsu.** 2008. Detection of naturally occurring enteroviruses in waters using direct RT-PCR and integrated cell culture-RT-PCR. J Virol Methods **149:**184-9.

42. **Shin, G. A., and M. D. Sobsey.** 2008. Inactivation of norovirus by chlorine disinfection of water. Water Res **42:**4562-8.

43. **Teunis, P. F., C. L. Moe, P. Liu, S. E. Miller, L. Lindesmith, R. S. Baric, J. Le Pendu, and R. L. Calderon.** 2008. Norwalk virus: how infectious is it? J Med Virol **80:**1468-76.

44. **Thurston-Enriquez, J. A., C. N. Haas, J. Jacangelo, K. Riley, and C. P. Gerba.** 2003. Inactivation of feline calicivirus and adenovirus type 40 by UV radiation. Appl Environ Microbiol **69:**577-82.

45. **Urakami, H., K. Ikarashi, K. Okamoto, Y. Abe, T. Ikarashi, T. Kono, Y. Konagaya, and N. Tanaka.** 2007. Chlorine sensitivity of feline calicivirus, a norovirus surrogate. Appl Environ Microbiol **73:**5679-82.

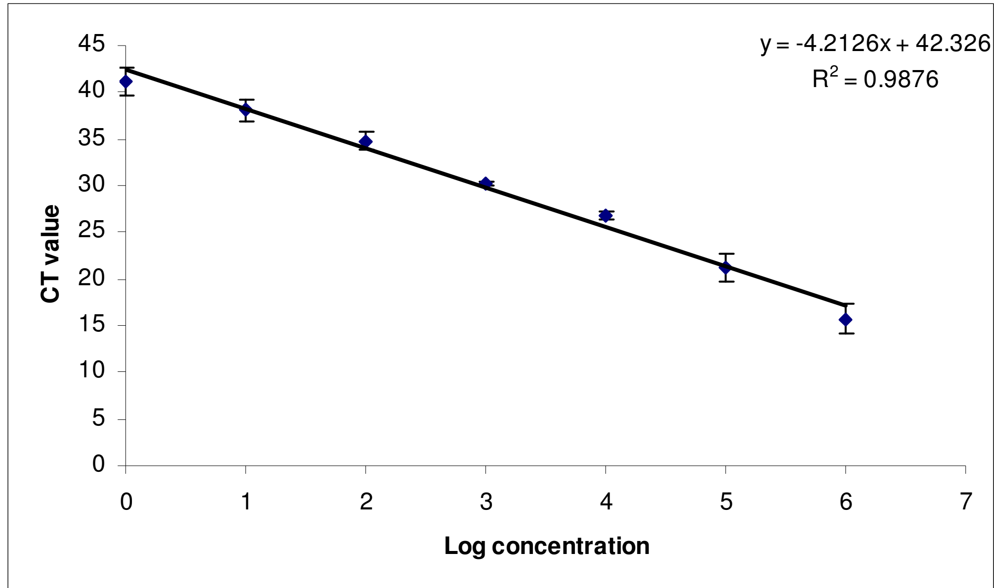
46. **Whitehead, K., and K. A. McCue.** 2010. Virucidal efficacy of disinfectant actives against feline calicivirus, a surrogate for norovirus, in a short contact time. Am J Infect Control **38:**26-30.

47. **Wobus, C. E., L. B. Thackray, and H. W. t. Virgin.** 2006. Murine norovirus: a model system to study norovirus biology and pathogenesis. J Virol **80:**5104-12.

APPENDIX

Figure 2.10 Standard Curves for a) MNV-1, b.) FCV, c.) HuNoV GII.4, d.) HuNoV GII.2 for RT-qPCR

A.



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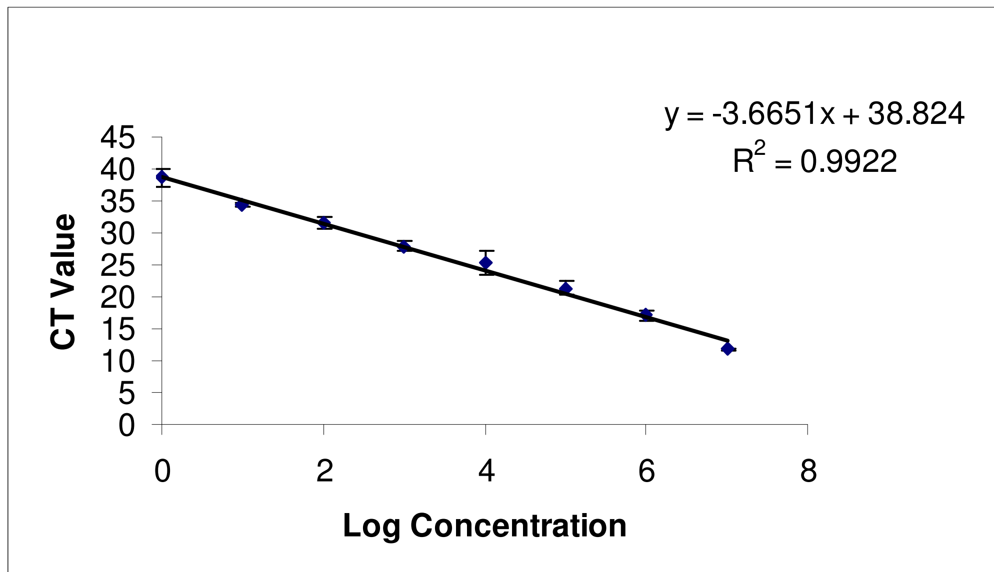

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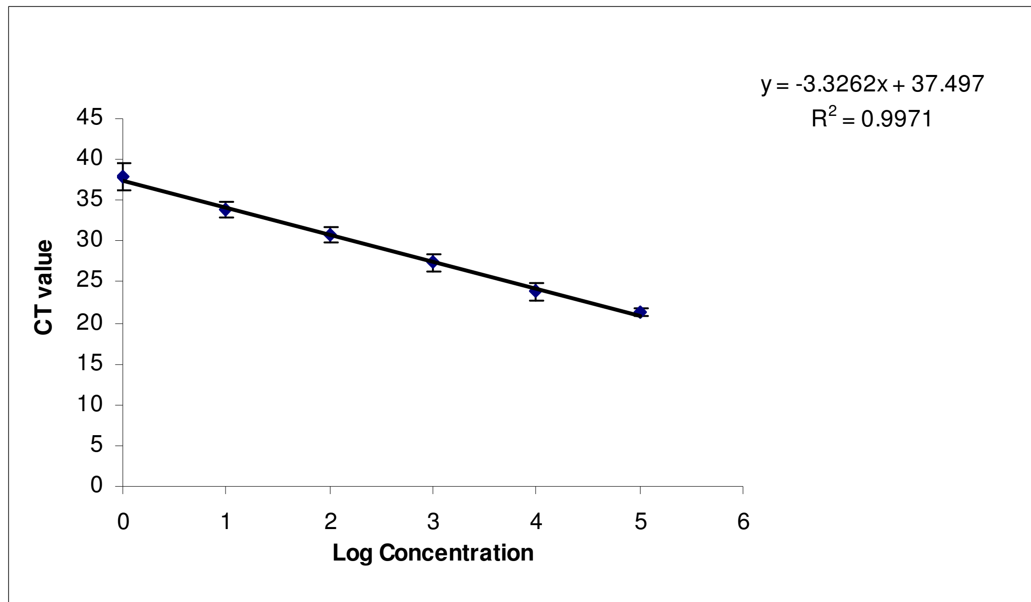

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