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Inactivation of human norovirus using chemical sanitizers ☆

David H. Kingsley [a,*], Emily M. Vincent [b], Gloria K. Meade [a], Clytrice L. Watson [b], Xuetong Fan [c]

[a] U.S. Department of Agriculture, Agricultural Research Service, Food Safety and Intervention Technologies Research Unit, Delaware State University, Dover, DE 19901, United States
[b] Department of Biological Sciences, Delaware State University, Dover, DE 19901, United States
[c] U.S. Department of Agriculture, Agricultural Research Service, Residue Chemistry and Predictive Microbiology Research Unit, Wyndmoor, PA 19038, United States

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ABSTRACT

The porcine gastric mucin binding magnetic bead (PGM-MB) assay was used to evaluate the ability of chlorine, chlorine dioxide, peroxyacetic acid, hydrogen peroxide, and trisodium phosphate to inactivate human norovirus within 10% stool filtrate. One-minute free chlorine treatments at concentrations of 33 and 189 ppm reduced virus binding in the PGM-MB assay by 1.48 and 4.14 $\log_{10}$, respectively, suggesting that chlorine is an efficient sanitizer for inactivation of human norovirus (HuNoV). Five minute treatments with 5% trisodium phosphate (pH ~ 12) reduced HuNoV binding by 1.6 $\log_{10}$, suggesting that TSP, or some other high pH buffer, could be used to treat food and food contact surfaces to reduce HuNoV. One minute treatments with 350 ppm chlorine dioxide dissolved in water did not reduce PGM-MB binding, suggesting that the sanitizer may not be suitable for HuNoV inactivation in liquid form. However a 60-min treatment with 350 ppm chlorine dioxide did reduce human norovirus by 2.8 $\log_{10}$, indicating that chlorine dioxide had some, albeit limited, activity against HuNoV. Results also suggest that peroxyacetic acid has limited effectiveness against human norovirus, since 1-min treatments with up to 195 ppm reduced human norovirus binding by <1 $\log_{10}$. Hydrogen peroxide (4%) treatment of up to 60 min resulted in minimal binding reduction (~0.1 $\log_{10}$) suggesting that H_2O_2 is not a good liquid sanitizer for HuNoV. Overall this study suggests that HuNoV is remarkably resistant to several commonly used disinfectants and advocates for the use of chlorine (sodium hypochlorite) as a HuNoV disinfectant wherever possible.

1. Introduction

Human noroviruses (HuNoVs) are now believed to cause the majority of foodborne illness in the United States (Scallan et al., 2011). Unfortunately, HuNoVs have not been reproducibly replicated *in vitro* (Duizer et al., 2004b; Herbst-Kralovetz et al., 2013) and while gnotobiotic piglets may have some promise (Cheetham et al., 2006), currently there are no practical animal models for *in vivo* studies. Human volunteer studies have been performed to assess inactivation techniques for HuNoV, but these studies are expensive to perform and logistically-complicated, limiting their use and the amount of data that can be obtained (Leon et al., 2011; Keswick et al., 1985). As a result, assessment of norovirus inactivation is typically evaluated using research surrogates, such as murine norovirus (MNV; Kingsley et al., 2007), feline calicivirus (FCV; Tree et al., 2005; Duizer et al., 2004a), and Tulane virus (Li et al., 2013). This is particularly true for the disinfectant research which principally utilizes FCV and MNV to assess probable inactivation by various sanitizers (Cromeans et al., 2010; D'Souza and Su, 2010; Fraisse et al., 2011; Kahler et al., 2010; Lim et al., 2010; Nowak et al., 2011; Predmore and Li, 2011; Tree et al., 2005; Urakami et al., 2007). While studies utilizing genetically-related viruses are of clear value, use of these research surrogates has some caveats. First, while surrogates may provide some idea about the sensitivity to a particular sanitizer, the data obtained only provides an estimate, or perhaps an educated guess, as to how human norovirus might respond to these chemicals. For example, FCV is not tolerant of low pH while MNV is tolerant (Cannon et al., 2006) and MNV is surprisingly more sensitive to alcohol than FCV (Sattar et al., 2011). Thus different research surrogates behave differently, making inferences about how HuNoV would react to different chemicals problematic. Secondly, most surrogate studies are performed using infected cell lysates prepared by pelleting cellular debris and filtering the supernatant. As a result, these lysate preparations are often complicated media mixtures containing fetal bovine sera and amino acids which may also react with sanitizers reducing their effective concentration. Furthermore while research surrogates can be purified and/or mixed with human fecal matter, the degree to which these experiments actually mimic human norovirus-contaminated stool is unknown.

Common sanitizers include chlorine (sodium hypochlorite), chlorine dioxide (ClO_2), peroxyacetic acid (PAA), hydrogen peroxide (H_2O_2), and trisodium phosphate (TSP; Na_3PO_4). Each has its own unique merits and drawbacks. Chlorine and ClO_2 are known to react with organic matter which can diminish active concentrations. Chlorine, but not ClO_2, is prone to formation of carcinogenic by-products, such as trihalomethanes,

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* Corresponding author. Tel.: +1 302 857 6406; fax: +1 302 857 6451.
E-mail address: david.kingsley@ars.usda.gov (D.H. Kingsley).

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when reacting with organic substances (Di Cristo et al., 2013). PAA and H_2O_2 have higher tolerance for organic materials and do not react with organic matter to form toxic residues (Herdt and Feng, 2009; De Luca et al., 2008). Also, PAA and its breakdown products (H_2O_2, oxygen and acetic acid) are not considered harmful. Trisodium phosphate, when dissolved, produces an alkaline solution but once washed away or neutralized, it is generally recognized as safe for treatment of food items. However management of phosphate laden wastewater could represent a substantial environmental challenge due to its propensity to induce eutrophication of freshwater ecosystems.

The inactivation mechanisms for virus disinfectants are not well delineated. For an enteric virus, there are two inactivation targets, the virus genome and the capsid. Chlorine is thought to target both capsid proteins and the RNA genome while for ClO_2, it is unclear if damage is only to the capsid or both the RNA and the capsid (Hirneisen et al., 2010; Sigstam et al., 2013). One important mechanistic difference may be that chlorine is prone to damaging the polypeptide backbone of capsid proteins while ClO_2 appears to predominately damage the amino acid side chains (Sigstam et al., 2013). H_2O_2 and PAA are strong oxidizers which may lead to oxidation of thiol groups in capsid proteins, and nucleic acid oxidation resulting in breakage of virus RNA (Finnegan et al., 2010). In solution, TSP has a high pH (~12). Evidence suggests that while HuNoV is probably quite tolerant of acidic conditions, basic conditions may cause the capsid to become unstable (Ausar et al., 2006).

A number of recent studies have sought to assess viral RNA damage by RT-PCR and other nucleic acid amplification methods as an indicator of inactivation. However, as judged by comparison with viable surrogates reductions in norovirus, qRT-PCR alone as a measure of human norovirus viability is thought to underrepresent inactivation (Shin and Sobsey, 2008; Park and Sobsey, 2011; Pecson et al., 2011). The technique described by Parshionikar et al. (2010) using propidium monoazide to block RT-PCR amplification of damaged RNA may prove better at assessing inactivation due to RNA damage assuming that capsid damage is sufficient to permit propidium to access norovirus RNA and the RNA damage is within the sequence region targeted for PCR amplification.

There have been a number of studies that have attempted to assess capsid damage in response to oxidation in the environment and in response to sanitizers. One technique involves biotinylation of oxidatively-produced carbonyl groups followed by avidin binding to exclude damaged capsids prior to RT-PCR (Sano et al., 2010; Tojo et al., 2013). Another technique utilized by Nuanualsuwan and Cliver (2002) used proteinase K and RNase A applied after inactivation by chlorine to destroy the inactivated virion and the genomic RNA of poliovirus (PV), hepatitis A virus (HAV), and feline calcivirus (FCV). In that technique, it was presumed that virion damage results in alteration of capsid protein structures resulting in a susceptibility to proteinase which destroys virus capsid integrity followed by release and digestion of the viral RNA genome by RNAse A. The loss of detectable virus RNA is then subsequently measured by qRT-PCR. It was noted that chemical inactivation of FCV, HAV and PV usually caused the loss of virus attachment to its homologous cellular receptor (Nuanualsuwan and Cliver, 2002, 2003).

Recently, an alternative method for evaluation of HuNoV inactivation, known as the porcine gastric mucin-magnetic bead (PGM-MB) binding assay, has been developed to assess the inactivation of norovirus (Dancho et al., 2012). It is known that the majority of HuNoV strains including GI.1 and GII.4 strains can bind to the porcine intestinal tract and to porcine gastric mucin (Tian et al., 2007, 2008, 2010), which chemically mimics the natural histo-blood group antigen receptor found in the human intestinal tract (Tan and Jiang, 2005; Tian et al., 2007). This mucin is commercially-available and can be conjugated to magnetic beads to extract norovirus from food and other matrices (Tian et al., 2008, 2011, 2012) for subsequent RT-PCR assay. Based on the premise that a damaged virus that cannot bind to receptor-like molecules is inactivated, this assay only extracts norovirus virions that are potentially infectious. This extraction/RT-PCR method has been used to define high pressure processing, ultraviolet light, and thermal treatment conditions which inactivate GI.1 norovirus (Dancho et al., 2012), as well as demonstrate that repeated freeze–thaw cycles have no discernible effect on HuNoV viability (Richards et al., 2012). In this study, we evaluate the efficacy of a number of common sanitizers used by the health care and food industries against HuNoV using the PGM-MB binding assay. We show that this technique has the potential to offer quantitative information about the inactivation of non-cultivable HuNoV.

2. Materials and methods

2.1. Norovirus stock

Norovirus stock was prepared from stool containing the GI.1 Norwalk strain of HuNoV from a volunteer study (Leon et al., 2011). The original titer of the stool was 10^8 genomic equivalents/ml. Stool was suspended and diluted 1:10 in distilled H_2O followed by centrifugation at 12,000 ×*g* for 20 min at 4 °C. Diluted stocks were passed through a 0.22-μM filter (Nalgene, Rochester, NY) and 1 ml aliquots were made and stored at −80 °C.

2.2. Sanitizer preparation and treatment

Chlorine (8.5% sodium hypochlorite) was obtained at a local market. PAA solution 39% (45% acetic acid/6% H_2O_2) was purchased from Sigma-Aldrich (St. Louis, MO). H_2O_2 (30%) was purchased from Sigma-Aldrich. TSP was purchased from Alfa-Aesar (Ward Hill, MA). ClO_2 in water was produced according to a method outlined in Standard Methods for the Examination of Water and Wastewater (American Public Health Association, APHA, 1998). The method involves the addition of hydrochloric acid (8%) to sodium chlorite in a sealed flask to generate ClO_2. A 2-l glass bottle containing 1-l cold water set within an ice bath was used to collect ClO_2. The ClO_2 was measured and was stored at 4 °C before use.

The effects of peroxide, PAA, ClO_2, and chlorine treatments were evaluated by mixing 25 μl samples of norovirus with 25 μl of diluted sanitizer followed by treatment with 10 μl of 1 M thiosulfate and/or adjusted to pH 7 using 2 N NaOH. Exposure times for were 1 min for chlorine, 10–60 min for ClO_2, 1–60 min for peroxide, 1 min for PAA, and 1–30 min for TSP. Trisodium phosphate treatments were performed in an analogous manner except that samples were neutralized with 2 N HCl with no thiosulfate treatment. Chlorine solution was diluted in 50 mM phosphate buffer, pH 6.5. All treatments were performed at ambient temperature (ca. 22 °C).

Actual (effective) sanitizer concentration in response to dilution in stool was evaluated. For safety and to facilitate measurement of effective sanitizer levels, separate norovirus stocks were heated to 99 °C for 15 min to inactivate the virus followed by mixing with different sanitizers (PAA, chlorine, ClO_2, and H_2O_2 in proportions analogous to norovirus/sanitizer treatments). Assessment of effective peroxide and PAA concentrations was performed using the peracid test kit #311 (Ecolab, St. Paul, MN) using the instructions from the manufacturer. Assessment of effective chlorine and chlorine dioxide was performed using the Hach DR890 kit following the manufacturer's instructions (Hach Inc., Loveland, CO).

2.3. PGM-MB binding assay

PGM-MBs were prepared as described previously (Dancho et al., 2012; Tian et al., 2008). In this study, the efficiency of capture for untreated HuNoV by the PGM-MB assay was >90%. After sanitizer treatment and neutralization of oxidizing chemicals and pH adjustment, NoV samples were diluted up to a final volume of 1 ml with PBS (without Ca^{+2} and Mg^{+2}) in a 1.5 ml microcentrifuge tube. Fifty μl