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"A Critical Review of Methods for Distinguishing Infectious and Non-Infectious Norovirus"

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1.0 Executive Summary

Reverse transcription quantitative PCR (RT-qPCR), the most widely used method to detect human noroviruses (hNoVs), is unable to discriminate between infectious and non-infectious virus particles. There is therefore a dilemma in assessing the risk to human health from samples detected as positive in RT-qPCR assays. This review provides a critical analysis of candidate methods which, when used in conjunction with RT-qPCR, may facilitate such discrimination. In the absence of an efficient cell culture based detection system for hNoVs RT-qPCR methods need to distinguish between intact infective particles, intact defective particles, degraded particles (consisting of capsid protein and virus RNA, herein referred to as ribonucleoprotein complexes (RNPs) and “naked” RNA. There are two major categories of methods that can aid in the determination of the origins of these signals: (i) those that measure viral capsid integrity; and (ii) those that measure viral genome integrity. The loss of capsid integrity has been studied in response to different inactivation methods as well as a part of the necessary steps in virus cell infection. Loss of capsid integrity can be accompanied by the exposure of the virus genomic RNA. Different approaches for determining capsid integrity and the measurement of RNA exposure as a marker for virus infectivity are critically reviewed. These are reviewed with reference to both virus inactivation studies and more fundamental studies regarding the mechanisms of capsid disassembly and RNA release during virus infection. Strategies for discriminating the origins of RT-qPCR signals are discussed together with long range RT-qPCR assays that may allow the detection of large genomic RNA fragments more likely associated with infectious particles. The detection of hNoVs is critically reviewed with particular regard to the application of capsid and genomic integrity assays to the draft European Committee for Standards (CEN) methods for the detection of hNoVs in foods and the environment.

2.0 Review Objectives

This critical literature review aims to identify potential approaches to assist in the interpretation of norovirus RT-PCR results and identify the following:

- The methods currently used to detect norovirus in food, the environment and clinical samples;
- The methods or approaches that could be capable of distinguishing infectious and non-infectious norovirus;
- The methods or approaches capable of assessing the integrity of norovirus capsid or RNA as a marker for infectivity; and
- The methods in the pipeline or on the horizon.

To illustrate the importance of this field, Hot et al., 2003, was able to detect enterovirus RNA in 60/68 samples of river water using RT-PCR, but only two of those samples were positive by culture. Studies on the long term survival of enteroviruses in groundwater have also shown that reductions in infectivity do not correlate with RNA survival as measured using RT-qPCR (De Roda Husman et al., 2009). This demonstrates the need for an improved understanding of the relationship between RT-qPCR signals and infectivity. The review particularly focuses on methods for the differentiation of PCR signals based on the integrity of the virus capsid and genomic RNA and builds on recent review articles examining methods for predicting virus infectivity (Cliver, 2009; Hamza et al., 2011; Rodriguez et al., 2009). Advances in these methods should aid in the development of RT-PCR assays that will be able to reliably predict infectivity in the absence of a culture system for hNoVs. The review also identifies research gaps, taking into account the practicality and complexity of applying such methods to foods or environmental samples. The findings of this review are intended to contribute towards the improved risk assessment of norovirus infection from foods and the environment.

3.0 Introduction

Human noroviruses (hNoVs) are recognised as the single most common cause of acute non-bacterial gastroenteritis in the industrialised world (Siebenga et al., 2009). These viruses belong to the Norovirus genus within the *Caliciviridae* family. Based on genome nucleotide sequence similarity, the Norovirus genus is further subdivided into different genogroups, and within each genogroup, specific genotypes. Genogroup II, genotype 4 (GII.4) viruses are the most common cause of human disease (reviewed by Patel et al., 2009). The single stranded RNA genome of hNoVs is approximately 7,600 nucleotides (nt) in length and protected within a virus capsid that is composed of 90 dimeric copies of a 60 kDa protein VP1, and a basic minor protein component (VP2) involved in the expression and stability of VP1 (Bertolotti-Ciarlet et al., 2003, Chen et al., 2006). The capsid shows T=3 icosahedral symmetry. Despite many attempts, scientists have been unable to reliably cultivate hNoVs *in vitro*, and there is no simple animal model that supports their replication (Duizer et al., 2004a). Even preliminary reports describing the culture of hNoVs using a complex, three-dimensional cell culture system has yet to be independently validated (Straub et al., 2011)

Human NoVs are detected most commonly using the Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) assay, or prior to the introduction of RT-qPCR, by conventional RT-PCR. Owing to their very low detection limits (theoretically, a single genome copy per RT-PCR reaction), RT-PCR-based approaches represent the methods of choice for detecting hNoVs in food and environmental samples, where the concentration of viruses in contaminated samples is usually quite low. However methods that are designed to detect viral nucleic acids are unable to distinguish between infectious and non-infectious virus, the latter of which might consist of defective virus particles and intact or degraded viral RNA. The reason for this is that RT-qPCR relies on the amplification and detection of small (ca 100b) nucleic acid sequences that may originate from either the much larger viral genomic nucleic acid (ca 7600 nt) associated with infectious particles, from detective virus particles having intact or partially intact genomes, and/or from degraded RNA

derived from inactivated particles. Furthermore, the scientific community has unequivocally demonstrated that the RNA associated with inactivated viruses remains detectable by nucleic acid amplification long after viral infectivity has been lost (Richards, 1999). In short, in the absence of a culture method or a suitable marker for infectivity, it is impossible to confirm that the detection of hNoVs by RT-qPCR is indicative of the presence of infectious virus. However it may be possible using the approaches discussed in this review to determine the origins of different RT-PCR signals and to provide more informed data for use in risk assessment.

4.0 Current methods used to detect hNoVs

4.1 Separation and concentration methods

Separation and concentration steps are a necessary prerequisite of virus detection methods as applied to food and environmental samples. Such virus concentration and purification schemes are designed to reduce sample volume and remove at least some of the matrix while simultaneously recovering most of the contaminating viruses. In order to achieve these goals, sample manipulations are undertaken that exploit the behavior of the viruses to act as proteins in solutions, to co-sediment by simple centrifugation when adsorbed to larger particles, and to remain infectious at extremes of pH or in the presence of organic solvents. Many virus concentration and purification methods use manipulation of pH and/or ionic conditions to favor virus adsorption to, or elution from, the food matrix. This is then followed by relatively low speed centrifugation after which the virus-containing phase (either precipitate or eluate) is recovered for further purification. Other steps in the virus concentration and purification process may include various forms of filtration (crude filtration and ultrafiltration), ultracentrifugation, precipitation (achieved through the addition of polyethylene glycol, organic flocculants, or by manipulation of pH), organic solvent extraction (to remove matrix-associated lipids), ligand-bound magnetic separation (using immunobeads or cationic particles), and/or enzyme pre-treatment (to break

down matrix-associated organic matter, particularly complex carbohydrates). In almost all instances, virus extraction is done by combining two or more of these steps in series (Jaykus & Escudero-Abarca, 2010; Stals et al., 2012).

Another important and alternative approach to virus concentration has focused on the use of binding ligands. For example, it has been demonstrated that magnetic bead-bound histoblood group antigens (HBGAs) (the putative host cell receptor for hNoVs) can be used for virus capture from environmental water samples (Cannon et al., 2008; Tian et al., 2012; Tian et al., 2011; Tian et al., 2010;). Porcine gastric mucin has been used in place of purified HBGAs in similar assays (Tian et al., 2008). Although antibody capture is also possible (Schwab et al., 2001), these methods remain limited owing to the absence of commercially available broadly reactive antibodies for the antigenically diverse hNoVs.

4.2 Nucleic acid purification

Following separation and concentration, viral RNA can be released from capsids and made available for amplification using a simple heat treatment (99^{o}C for 1 min or more). This is, however, rarely done because, even with the best virus concentration method, residual matrix-associated components persist and oftentimes interfere with nucleic acid amplification. Therefore, an efficient nucleic acid extraction step is critical. The science of RNA extraction has developed rapidly over the last decade and what was once a complex procedure is now relatively simple and reliable (Jaykus & Escudero-Abarca, 2010). Most investigators currently use methods combining guanidinium thiocyanate solution to denature proteins and silica to bind released nucleic acids (Boom et al., 1990). These methods are available in kits and have recently been automated by a number of diagnostics manufacturers.

4.3 RT-qPCR detection methods for hNoVs

The inability to cultivate hNoVs has meant that clinical assays have predominantly relied on electron microscopy, ELISA assays, RT-PCR and RT-qPCR as detection methods. Comparative analysis of these methods for clinical outbreak investigations has estimated their sensitivities as 17%, 86% and 100%, respectively (Fisman et al., 2009). Owing to their relatively lower detection limits, RT-PCR and RT-qPCR are the most commonly used methods for detecting hNoVs, at least in food and environmental samples where the level of virus contamination is quite low. Originally RT-PCR was based on random primed reverse transcription of extracted RNA followed by specific PCR under stringent conditions (1.5-2.5M $MgCl_2$), with confirmation of the product identity by size measurement using agarose gel electrophoresis. Gel-based assays have now largely been replaced by the use of one step RT-qPCR which allows for detection and amplicon confirmation in a single reaction. One step RT-qPCR involves the use of the forward PCR primer to act as a specific primer for reverse transcription prior to qPCR, allowing the entire amplification to be done in a single tube. Typically qPCR uses less stringent PCR amplification conditions (3-5mM $MgCl_2$) in conjunction with 5' nuclease-based hybridisation probes to measure an increase in fluorescent signal concomitant with amplicon accumulation at each PCR cycle. In general, RT-qPCR is more sensitive than conventional RT-PCR, and less liable to produce false positive results due to PCR contamination. Broadly reactive RT-qPCR primer sets for GI and GII hNoVs as well as hepatitis A virus (HAV) have been described by a number of investigators (Jothikumar et al., 2005a; Jothikumar et al., 2005b; Kageyama et al., 2003). The European Committee for Standards (CEN) have utilised similar broadly reactive primer sets in the development of standardised methods for the detection of viruses in complex matrices such as food and water in support of food safety legislation.

In order to understand the capability of these methods for the detection of environmental hNoV RNA (as compared to intact particles that may be presumed infectious), it is important to understand the basis of the extraction procedure. The proposed draft CEN standards recommend different methods for extracting and concentrating viruses from different foods and the environment (Table 1). These

methods may result in either the recovery of RNA, intact virus particles, or both, from the test matrix. RNA is further purified and concentrated by treating samples with guanidinium isothiocyanate solution to lyse the virus capsid and capture RNA by binding to silica as described above. RNA may then be eluted and tested in RT-qPCR. The sum total of these sample manipulations is technically demanding and probably not suitable for routine microbiological laboratories, but rather require more specialised testing services.

Table 1. Key steps in the proposed CEN virus extraction methods and their potential to co-purify RNA from different matrices prior to the final guanidinium isothiocyanate/silica RNA binding and elution step.

Application	Sample size	Concentration step	pH	Purification of virus	Purification of RNA
Water	0.3-5l	+ve charged filter	7.3	Yes	Yes
Food surfaces	Swab 100 cm2 (max)	None	9.5	Yes	Yes
Fruit & salad vegetables	Chopped samples (25g)	PEG precipitation	>9.0	Yes	Yes
Bivalve molluscan shell fish	10 X Digestive glands (2g)	None	Not stated	Yes	No

All of methods outlined above in Table 1, with the exception of the shellfish method, have potential to recover RNA as well as intact hNoV particles. These methods therefore cannot determine if the resulting RT-qPCR signals are derived from intact virus particles or residual RNA from degraded virus particles. However, shellfish do not significantly bioaccumulate purified RNA (Dancer et al., 2010) and it is possible that the shellfish detection method predominantly results in the detection of hNoVs from intact particles, although this has not yet been verified.

When done under optimal conditions (e.g. a clean matrix, high recovery efficiency from the extraction method, etc), RT-qPCR allows the highly sensitive and specific detection of target RNA sequences. According to the proposed CEN standard, 5' nuclease assays "*obviate the need for additional amplification product confirmation steps post PCR".* However, in a number of cases, studies have demonstrated that RT-qPCR signals generated (especially those obtained close to the limit of detection)

in these assays cannot subsequently be confirmed by cloning and sequencing (Boxman et al., 2011; Mattison et al., 2010) calling into question whether these are true positives. In fact, when such signals have been investigated in similar (but not identical) RT-qPCR assays, false positive results owing to PCR artefacts and/or PCR contamination have been reported (Baert et al., 2011; Stals et al., 2011; Stals et al., 2009). It appears possible that in some cases, the reduced stringency of primer annealing in the RT-qPCR reverse transcription and amplification steps might lead to non-specific amplification followed by a low level of probe hybridisation resulting in background signal.

Further studies are required In order to provide unequivocal differentiation of true positive results from any background RT-qPCR signals in the absence of a confirmatory test. However confirmatory analysis is made difficult by the lack of alternative conserved PCR primers for hNoVs. More recent attempts to assess hNoV risk from shellfish have shown significantly higher levels of RT-qPCR signal associated with outbreaks compared with those not associated with outbreaks (Lowther et al., 2012), suggesting that differentiation such as this may be particularly important in risk assessment.

MIQUE guidelines (Minimum Information for Publication of Quantitative Real-Time PCR experiments (Bustin et al., 2009) treat Cq values (the threshold PCR cycle at which product first accumulates) of greater than 40 as suspect. Experiments modelling the limit of detection in real-time PCR suggest a Cq cut-off value of 38 with 3 replicates per sample corresponding to 8-12 copies (Burns & Valdiva, 2007). Cq values for naturally contaminated environmental and food samples frequently go unpublished in the peer-reviewed literature, but when provided, are often in the range of 32-38 or more (Baert et al., 2011; Boxman et al., 2011; Tian et al., 2012).

However, the determination of copy number associated with a specific Cq value is dependent upon the standard used. It also depends on the sample matrix, and there is evidence that amplification efficiency is reduced in the presence of matrix-associated inhibitory compounds, particularly when the template copy number is low (as is the case for most naturally contaminated food and environmental samples)

(Sair et al., 2002). Increased Cq values may also result from induced fluorescence associated with the matrix (Baert et al., 2011). It is clear that there is a need for international consensus on the appropriate standard for use for genome quantification based on RT-qPCR Cq values, and the recent development of lenticule standards (Hartnell et al., 2012) for use in the proposed CEN standard should be very useful in this regard.

4.4 Alternative RNA based detection methods

Alternative RNA-based detection methods have also been applied to the detection of hNoVs in faecal samples and foods. These include nucleic acid sequence based amplification (NASBA) (Houde et al., 2006; Lamhoujeb et al., 2009; Patterson et al., 2006) and reverse transcribed loop mediated isothermal amplification (RT-LAMP) (Fukuda et al., 2008; Fukuda et al., 2006). Like RT-qPCR, there is no reason to believe that these methods, without further refinement, can be used to discriminate between infectious and non-infectious hNoVs.

4.5 Enzyme Linked Immuno-Sorbent Assays (ELISA) assays

In general, most ELISA assays consist of mixtures of antibodies derived from different hNoV virus-like particles VLPs, (*in vitro* synthesised hNoV capsids comprised of the major capsid protein, P1). These ELISA assays contain a combination of antibodies capable of recognising a broad range of antigenically diverse hNoVs. However, commercial ELISA assays cannot distinguish between infectious particles, empty capsids and free hNoV antigen that may be present in infected individuals (Hardy et al., 1995) and thus signal from ELISA assays does not correlate with those obtained by RT-qPCR (Costantini et al., 2010) Direct comparison of commercial ELISA kits with RT-PCR assays has shown that although ELISAs can detect the majority of cases and may be useful as a preliminary screening tool, they are relatively insensitive, do not detect all genotypes and can therefore result in false negative results (Bruggink et al., 2011; Burton-MacLeod et al., 2004; De Bruin et al., 2006; Kele et al., 2011). ELISA assays typically have detection limits of around 10^5 particles per reaction and are thus insufficiently

sensitive for the direct detection (without prior cultural enrichment or pathogen concentration)of microbial contamination in foods and the environment.

ELISA assays for hNoVs are commercially available from R-Biopharm AG (Germany) and Oxoid (UK). R-Biopharm manufacture RIDA®SCREEN and RIDA®QUICK assays. RIDA®QUICK is a flow-through immunochromatographic test strip and is the only ELISA with official FDA approval. However, owing to its limited sensitivity, the method can only be used for preliminary identification of an outbreak and not as a diagnostic tool. Oxoid manufacture the IDEIA enzyme immunoassay kit. Comparison between these test kits with RT-qPCR results suggests that the RIDA®SCREEN and RIDA®QUICK assays are more sensitive than the IDEIA assay (Kirby et al., 2010).

4.6. Electron Microscopy (EM)

The Norwalk virus was the first norovirus identified and originally detected by EM in stool suspensions derived from children involved in an outbreak of acute nonbacterial gastroenteritis (Kapikian et al., 1972). Since that time, EM has continued to be used to detect hNoVs in clinical specimens. In this application, EM has remained mostly a research tool, as clinical laboratories do not have routine access to the expensive EM equipment. The detection limit of EM ranges from 10^6-10^7 virus particles and about one $\log_{10}$ lower if used along with fluorescently-labelled antibodies. EM allows the direct visualisation of virus particles and can distinguish intact particles from “empty” capsid shells that typically appear as ghosts with an electron-dense centre of accumulated stain and a bright outer periphery. Although the technique is relatively insensitive, it remains useful for the detection of emerging viral pathogens but shows little utility for detection of contamination in food and environmental samples.

5.0 The problem of defining virus ‘infectivity”

Both the virus capsid and its genome are required for infectivity. Although viral RNA can be infective *in vitro* (Bachrach, 1959; Holland et al., 1960), the virus capsid is necessary to protect the genome from degradation in the human host and in the

environment. Hence, loss of integrity of the virus capsid usually results in loss of cellular attachment, receptor binding and/or exposure of the viral nucleic acid to nucleases present in the environment, all of which will result in a loss of infectivity. In a similar manner, irreversible changes to the integrity of viral RNA will mean that the infection cycle cannot be completed.

However, in virus suspensions obtained from either animal cell culture lysates or natural infections, not all particles are actually able to produce infection if exposed to susceptible cells or hosts. This is known as the particle-to-plaque forming unit (pfu) ratio, and is a measure of the fraction of virus particles in a given sample that can complete an infectious cycle. For many animal viruses, these ratios can be as high as 10,000:1. A high particle-to-pfu ratio is sometimes caused by the presence of defective particles with capsids having minimal damage (which is indistinguishable by EM) or genomes that are in some way damaged or incomplete. However, an alternative explanation is the possibility that a single virus, while having the capability of initiating infection, may fail to complete the process due to host defenses or simply because one or more steps in the infection process fails to be successfully completed. Studies of poliovirus (PV) suggest that although defective particles remain infectious and their RNA is successfully released into cells upon infection, subsequent steps in the replication cycle are blocked (Brandenburg et al., 2007).

An additional consideration is the fact that in natural suspension, hNoVs have a tendency to aggregate. Virus aggregation appears pH dependent and may influence the results of infectivity assays (Langlet et al., 2007). Aggregation of infectious particles changes the dose-response relationship, and hence virus infectivity. Specifically, in an infectivity assay, a single infectious virus can theoretically infect a single susceptible cell; similarly, an aggregate that may contain from two to hundreds of infectious viruses, will also infect a single susceptible cell. This phenomenon was illustrated by (Teunis et al., 2008) who estimated Norwalk virus infectious dose in humans based on aggregation status. In this case the 50% infectious dose (ID_{50}) for single virus particles was about 18, but was about 10-fold lower (2.6) when taking into account virus aggregation.

Both of these issues are probably responsible for the fact that, when one extrapolates cultivable virus concentration in high titre samples by comparing RT-qPCR Cq values to standard curves, the virus concentration is usually 10 to 100-fold more than that obtained using comparative infectivity assay (Li et al., 2011). Unfortunately, both particle-to-pfu ratio and virus aggregation status are difficult to characterize. In fact, for any single, unique virus suspension, these values depend on a variety of factors, including the type of host cell, the suspension matrix, and even when in the infection cycle the suspension was harvested. This is not to mention the fact that the proportion of a virus suspension consisting of intact, naked genomic RNA, degraded genomic RNA, incomplete virus particles, and complete (infectious) virus particles, is unknown and likely impacted by the same factors. In short, defining an "infectious" virus is in itself, complicated. This reality must be taken into account when attempting to evaluate "infectivity" using any potential assay method.

6.0 Virus integrity and virus infectivity

Nonetheless, studies on virus capsid and genomic stability have provided the basis for our understanding of their contribution to viral infectivity. These findings may be exploited to identify candidate treatments that are able to discriminate infectious from non-infectious virus particles.

Historically most data regarding the stability of the capsid and the genome of RNA viruses, and their relationship to infectivity, has been collected during the study of the heat inactivation of pathogenic viruses and vaccines. Capsid integrity is also essential for infectivity and of interest to those studying viral infection since virus uncoating and the egress of viral RNA from virus particles following heat inactivation appears to mimic the early stages of viral infection (Levy et al., 2010). Most studies have examined three important picornavirus pathogens, i.e., rhinoviruses, poliovirus (PV), and foot and mouth disease (FMDV) viruses, and to a lesser extent, Cocksackie B virus and hepatitis A virus (HAV). However the *Picornaviridae* are structurally distinct from the *Caliciviridae*, having a capsid consisting of 60 copies of

each of four coat proteins (VP1, VP2, VP3 and myristoylated VP4) arranged in T=1 icosahedral symmetry.

The heat inactivation of picornaviruses is a biphasic process. At low temperatures (<40ºC) inactivation is thought to proceed through the degradation of viral nucleic acid whilst high temperature inactivation (>40ºC) effects virus protein (Dimmock, 1967; Hiatt, 1964). RNA extracted from either heat inactivated FMDV (Bachrach 1959), or PV or coxsackievirus B-2 virus particles (Larkin & Fassolitis, 1979; Nuanualsuwan & Cliver, 2003b) remained infectious, showing that heat treatment acted on the virus capsid protein. RT-qPCR signals derived from hNoVs subjected to heating at 100ºC for 1 minute remain relatively unaffected (Δ Cq = 1.9) (Duizer et al., 2004b). Capsid stability and virus infectivity is not only impacted by temperature, but is dependent on differences in virus structure, pH, the particular strain, and purity of the test suspension (Doel & Baccarini, 1981; Salo & Cliver, 1976; Terry et al., 1983). Not surprisingly ionic strength influences capsid stability dependent on the selected virus (Bachrach, 1965; Dimmock; 1967; Lee & Gillespie; 1973; Wetz & Kucinski, 1991. Since virus inactivation studies are rarely performed under the same conditions factors such as these may complicate the direct comparison between different viruses unless compared under identical conditions (Park and Sobsey, 2011; Siegl et al., 1984).

In the *Picornaviridae,* heat inactivation at high (>40ºC) temperatures is accompanied by reduction in sedimentation co-efficient of the virus particle. In PV this results in a transition from mature virions (160S particles) via a 135S intermediate to 80S "empty" capsid shells, with simultaneous release of VP4 and RNA. The transition between 135S particles, the simultaneous release of VP4 and RNA, and the formation of 80S particles takes place between 50ºC and 56ºC. This may be indistinguishable in heat inactivation studies and appears as a continuous transition concomitant with loss of infectivity (Hinuma et al., 1965). 135S particles may also be formed by heat treatment at 37ºC for 30 min as referenced by (Curry et al., 1996). The 135S particles show externalisation of VP4 and the amino terminal extension of VP1. They also demonstrate reduced (10^3-10^5 fold) infectivity for their HeLa host

cells, and altered host cell specificity, making Chinese Hamster Ovary and murine L cells permissive to transient infection. Infectivity of 135S particles is unaffected by RNase treatment, but the amino terminal of VP1 is more sensitive to protease (Curry et al., 1996). Further heating of 135S particles results in the loss of VP4 and transition to 80S "empty" capsid shells with expulsion or egress of part or all of the genomic RNA, resulting in 80S e (early) or 80S l (late) particles, respectively. Viral RNA egress is thought to occur through a small pore in the capsid near a twofold symmetry axis (Bostina et al., 2011). The RNA within non-infectious 80S e particles may be RNase resistant and become susceptible following egress from the particle to form the 80S l empty capsid (Breindl, 1971; Levy et al., 2010).

In contrast, the capsid of the more temperature sensitive FMDV virus appears to dissociate into individual pentamers and mutations affecting FMDV capsid stability map to amino acids located within the interfacial pentameric sub-units (Mateo et al., 2003). Cellular uptake of the FMDV surrogate equine rhinitis A virus, human rhinovirus, and the hNoV surrogate FCV F-9, all proceed through clathrin–mediated endocytosis resulting in the dissociation of the capsid in the acidic pH of the endosome (Groppelli et al., 2010; Stuart & Brown, 2006). In contrast, the cell entry of PV and the norovirus surrogate MNV-1 is mediated through a non-clathrin, non-caveolae pathway (Gerondopoulos et al., 2010). Both enterically infecting PV and MNV-1 are more acid resistant than respiratory infecting FMDV and the less closely related, Vesivirus, surrogate FCV F-9 (Cannon et al., 2006; Wetz and Kucinski, 1991). This is reflected in differences in the cell entry mechanisms and capsid dissociation, since the FCV-F9 and FMDV capsids do not need to withstand transit through the acidic pH of the stomach. Thus the capsids of the commonly used hNoV surrogates MNV-1 and FCV-9 dissociate quite differently.

Low temperature virus inactivation (<40ºC) can result from single strand breaks caused by the enzymatic degradation of genomic RNA by internalised capsid enzymes including ribonuclease (Denoya et al., 1978a; Denoya et al., 1978b; Scodeller et al., 1984) or polymerase (Newman et al., 1996). Although the relevance of this phenomenon to hNoVs is unknown, it does represent a mechanism of "self

inactivation" and may explain why some viruses "die off" over time. In this case, the viral RNA in the capsid may become degraded and yet may still be detectable by RT-PCR. Additionally the external proteolytic cleavage of the virus capsid has also been reported with concomitant release of RNA (Herrmann and Cliver, 1973).

7.0 Candidate methods for discriminating infectious from non-infectious hNoVs

As stated above, hNoVs cannot be grown outside of their human hosts and none of the methods discussed so far (i.e. EM, ELISA, and RT-qPCR) are definitively able to discriminate between infectious and non-infectious virus. There are two major reasons why scientists need reliable means by which to discriminate hNoV infectivity status: (i) to support studies to identify hNoVs in naturally contaminated samples; and (ii) to support studies to understand virus inactivation, including characterization of the efficacy of both traditional and novel control measures. This "infectivity dilemma" has been of increasing interest to scientists for the last decade, and alternative methods for predicting virus infectivity based on molecular approaches have been investigated. These methods fall into two major classifications: (i) those based on examining the stability of the virus genome; and (ii) those based on examining capsid integrity. The following sections (7.1-7.3) discuss these candidate methods.

7.1 Measurement of genomic integrity

The detection of small genomic target fragments using RT-qPCR may not indicate the presence of infectious virus for two reasons. The first reason is that naked viral RNA can persist long after a viral capsid has been destroyed. The second reason is that even though a single strand break occurring anywhere in the viral genome, will render a virus particle non-infectious, the same break cannot be detected by RT-PCR if the target region for amplification remains intact. To account for this problem, it has been suggested that measurement of the overall integrity of genomic RNA could provide a useful marker for infective particles.

For some inactivation strategies (e.g. ultraviolet light), random damage may occur anywhere on the genome, resulting in loss of viral infectivity. In instances such as these, RT-qPCR will underestimate infectivity losses because the resulting amplicons may originate from genome fragments rather than whole genomic viral RNA. Pecson et al., (2011) reported a theoretical framework to relate viral infectivity with genome damage using qPCR. Assuming single hit inactivation kinetics, and a homogenous sensitivity of the viral RNA to UV, these investigators used a Poisson distribution to demonstrate proof-of-concept that their mathematical approach could predict the loss of infectivity for bacteriophage MS2. However, upon further investigation, they observed that genome regions actually demonstrated heterogeneous sensitivities to UV, thus deviating from the Poisson assumption. They noted that a way around this problem would be to do multiple amplifications from multiple genomic regions, although such an approach is cumbersome. It is important to note that this sort of method would only be useful in instances where the inactivation occurs via single genomic lesions. Further, there is evidence that in animal viruses, UV light may initially target the virus capsid (Nuanualsuwan & Cliver, 2003a). As noted above fragmentation of viral nucleic acids within capsids may also occur as a result of internalised enzymatic activity.

Although it is possible to amplify near full-length hNoV genomes (Kostela et al., 2008), amplification efficiency decreases with fragment size, thus making amplification of full length genomic RNA relatively insensitive. By using long range reverse transcription complimentary to the virus 3'end, followed by a more efficient small fragment RT-qPCR targeting the 3' end of the cDNA, it is possible to design a more efficient PCR-based method to detect whole viral genomes. This approach has been used to examine the integrity of the MNV-1 and hNoV genome following high temperature (72ºC) heat treatment (Wolf et al., 2009) MNV-1 genomic RNA was relatively insensitive to heat treatment as measured by RT-qPCR in comparison to infectivity assays irrespective of the size of the target amplicon. In contrast, loss of infectivity following UV exposure was accompanied by a greater loss of RT-qPCR signal from the 3'cDNA end relative to the 5' end, showing that larger RT-PCR targets were more sensitive to UV i.e. larger RT-qPCR amplicons are more

susceptible to UV induced scission and more likely associated with infectivity. It would be of interest to apply this assay to low temperature virus inactivation studies to investigate the influence of any internalised enzymes on MNV-1 and hNoV genomic integrity and infectivity.

7.2 Measurement of capsid Integrity or "virolysis"

Owing to the importance of the virus capsid in infectivity, the measurement of capsid integrity or "virolysis" may represent an alternative marker for virus infectivity in the absence of a culture model for hNoVs. Virolysis results in exposure of the viral RNA due to degradation of the viral capsid. This approach was first demonstrated for FMDV prior to the invention of PCR (Terry et al., 1983). In these studies, exposure of RNA following heat treatment was measured spectrophotometrically following RNase treatment. Results were in close agreement with the loss of integrity of the 140S native infective particle resulting in typical sigmoidal melting curves.

The spectrophotometric method requires large quantities of virus and more sensitive detection of exposed RNA can be obtained by combining RNase treatment with RT-PCR. A number of advantages exist in the application of this approach to non-cultivable viruses including hNoVs. Firstly, only small quantities of relatively dilute samples are required; secondly, the virus can be examined in different matrices; thirdly, the data can be directly compared to that obtained from surrogate viruses using spiking experiments; and fourthly, the data can be compared with that obtained in plaque assays using surrogate viruses. Absolute copy number determination is not required since experiments measure relative differences in RNA exposure. However, it is worth noting that it is not possible to compare the absolute copy number obtained from virolysis to that of the plaque assay because of multiplicity of infection and aggregation issues, although relative reductions should be comparable

A further consideration in the interpretation of RT-qPCR data from RNA exposure studies is the mechanism of RNA exposure and its relationship to infectivity. In simple heat inactivation studies, RNase treatment and RT-qPCR provide an indirect method of measuring protein denaturation, with the assumption that exposed RNA

would no longer be infectious. However, there are at least three model mechanisms by which RNA can be exposed following heat treatment that may reflect differences in virus capsid dissociation discussed above. Firstly, a simple “all or nothing” model (Ros et al., 2006) in which the entire genome is exposed instantaneously, with each exposure event corresponding to the inactivation of one particle. This is the most commonly presumed model. However, a second possibility, an extrusion model, assumes that viral RNA is progressively exposed starting from one end of the genome and working to the other end (Levy et al., 2010). This extrusion model is perhaps consistent with the observations of increased sensitivity of the 5’ and 3’ ends of PV to chlorine dioxide in relation to infectivity, as measured by RT-qPCR (Simonet and Gantzer, 2006). It may also describe the increased sensitivity of the 5’ non-translated region of HAV to chlorine and chlorine dioxide (Li et al., 2002; Li et al., 2004), Thirdly, a partial exposure model, in which a particle is damaged but only a fraction of the genome is exposed, is consistent with the random dissociation of virus particle sub-units (Topping et al., 2009).

These different RNA exposure models may describe fundamental mechanisms of capsid dissociation described above, and can certainly impact the interpretation of RT-qPCR data relative to virus infectivity. For this reason, different approaches have been taken to assessing the integrity of the virus capsid as a proxy for infectivity assay.

7.2.1 Measurement of capsid integrity using RT-qPCR with Proteinase K and RNase A pre-treatment

Nuanualsuwan & Cliver, 2002; first published this method in their attempt to correlate conventional RT-PCR data with infectivity. In preliminary studies, these investigators observed that, although RNase pre-treatment reduced RT-PCR signals associated with viruses that had undergone complete inactivation by heat, the pre-treatment alone failed to completely abolish RT-PCR signals. This was considered owing to residual protection of RNA by the partially degraded capsid. Similar observations of the formation of RNase resistant RNA-protein complexes (described as

ribonucleoprotein complexes (RNPs)) were originally made for PV following heat treatment prior to the invention of PCR (McGregor & Mayor, 1971; McGregor & Mayor, 1970; McGregor, 1968). In order to overcome this problem Nuanualsuwan and Cliver introduced a simultaneous proteinase K and RNase A digestion step, the former of which was intended to degrade the capsid of damaged virus particles (without destroying intact particles), and in so doing abolish (> 2-3 $\log_{10}$ reduction) residual RT-PCR signals. Taken together, this provided a more direct comparison between RT-PCR data and the loss of virus infectivity.

This combined enzymatic treatment (ET) was extensively studied in conjunction with RT-qPCR for the small RNA bacteriophage MS2 (Pecson et al., 2009). Using PCR amplicons covering the entire MS2 genome, these investigators showed that:

(i) RT-qPCR signals associated with inactivated viruses could be reduced significantly (up to 5.2 $\log_{10}$) but not completely abolished by preceding the amplification with ET;

(ii) that reductions in the RT-qPCR signal obtained depended on the method of virus inactivation; and

(iii) that different genomic regions may confer different levels of protection on the viral genome.

In this case, decreases in virus infectivity were always accompanied by dose-dependent decreases in ET-qRT PCR signals. In contrast, studies using MNV-1 in conjunction with ET RT-qPCR did not show similar reductions in RT-qPCR signals following heat treatment of the virus at 80ºC for 150s or greater (Baert et al., 2008) Diez-Valcarce et al., 2012). However, RNA signals from high-pressure treated MNV-1 were reduced in comparison to untreated controls using ET in conjunction with conventional RT-PCR, indicating that high pressure results in damage to the MNV-1 virus capsid (Tang et al., 2010). Poschetto et al., (2007) also noted that RNase and proteinase K pre-treatments did not reduce the RNA concentration of FCV F-9 exposed to sodium hypochlorite, even though infectivity assay demonstrated virus inactivation. These data, however, contrast with that of (Nuanualsuwan & Cliver,

2003a) who showed that hypochlorite effectively abolished RNA signals from treated FCV F-9 without enzymatic pre-treatment.

Capsid integrity has also been investigated using combined proteinase K and RNase treatment in conjunction with isothermal nucleic acid sequence-based amplification (NASBA). In order to avoid inhibition of RNase activity by proteinase K Lamhoujeb et al. (2008) separated these enzymatic steps by first incubating with high concentrations of proteinase K, then inactivating the proteinase K activity, and finally digesting exposed RNA with RNase A. Results for heat inactivation of a 20% (v/v) stool suspension derived from a GII, 4-infected patient showed that high temperature-long time heat treatments (e.g. 72 °C for 45 to 75min with high levels of proteinase K and RNase) were required in order to eliminate NASBA signals.

7.2.2 Measurement of capsid integrity using RT-qPCR with RNase pretreatment treatment alone

Over time, it became apparent that the use of combined proteinase K and RNase is difficult to control and reproduce for a number of reasons. Firstly efficient proteinase K digestion requires calcium ions (1mM) for enzyme stability and the prevention of autoproteolysis. Secondly, the enzyme is capable of degrading native proteins that may include virus particles (Mormann et al., 2010) and thirdly the enzyme digests RNase A and can therefore prevent RNase activity (Wiegers & Hilz, 1971). In fact, proteinase K is now widely used in nucleic acid extractions for the prevention of RNase activity. However it remains possible that, depending on the relative activity of each enzyme, residual RNase activity might persist in the presence of proteinase K, making it difficult to interpret data obtained from this approach.

Studies in which RT-qPCR has been preceded by RNase treatment alone have used either RNase A or RNase ONE. The latter enzyme is more readily inactivated and has reduced substrate specificity compared with RNase A. Using this approach and the RNase ONE enzyme, Topping et al., (2009) measured the destruction of the virus capsid (virolysis) following different processes as applied to hNoV and the

cultivable feline calicivirus vaccine strain (FCV F-9) as a surrogate. Results obtained following both heat and disinfectant (available chlorine) treatments demonstrated good agreement between the maximum observed reductions in RNase RT-qPCR signals and loss of infectivity for FCV. Data for FCV F-9 following heat treatment also shows that infectivity measured by plaque assay results in greater $\log_{10}$ inactivation owing to the lower dynamic range of the RNase ONE capsid integrity assay.

When the developed method was applied to various GII.4 hNoV strains present in dilute (1% v/v) stool suspensions, all strains behaved similarly and appeared significantly more resistant to both heat and chlorine than was FCV F-9. However, when FCV F-9 was spiked into a stool suspension, its resistance to virolysis increased significantly. This suggests that the increased capsid resistance observed for hNoVs resulted from protection by the natural faecal matrix (Nowak et al., 2011a; Topping et al., 2009). Similar studies using GII hNoVs in experimentally contaminated foods have showed much the same (Mormann et al., 2010). Likewise, Scheilke et al., (2011) used RNase A pre-treatment when investigating heat treatment for the inactivation of hepatitis E virus in a suspension of naturally contaminated boar liver. Data for the maximum virus reductions (>99.9%) calculated using RT-qPCR preceded by RNase pre-treatment were in accordance with the reported values for conditions resulting in loss of infectivity in culture assays.

Recent data using the RNase ONE approach for MNV-1 have only shown small but significant RNA exposure in comparison to loss of infectivity (Diez-Valcarce et al., 2011; Li et al., 2012). This contrasts with data from heat inactivation studies on a broad range of RNA viruses (including PV, HAV, rhinovirus, FMDV, FCV F-9, hepatitis E, and the bacteriophage MS2) all of which show significant exposure of RNase-sensitive viral RNA following heat treatment in conjunction with loss of infectivity and capsid integrity. The reason for this apparent difference in behaviour for MNV-1 is not known. Differences may result from the slower heating and cooling conditions used in these studies, perhaps resulting in the norovirus equivalent of non-infectious 135S particles, degradation of RNA, or the re-association of capsid protein with RNA during the cooling step to form RNase resistant RNPs (McGregor &

Mayor, 1968). It could also be due to a more fundamental difference in the capsid structure and behaviour of MNV-1.

7.2.3 Measurement of capsid integrity using "integrated" RT-qPCR approaches

Integrated approaches measure the affinity of virus particles for receptors involved in cellular attachment or other virus binding ligands, including antibodies, carbohydrates, or negatively charged (cationic) magnetic particles (Li et al., 2011; Li et al., 2012; Nuanualsuwan & Cliver, 2003a). These methods are also intended to discriminate infectivity status based on the integrity of the viral capsid, assuming that non-intact capsids would not be able to bind to the appropriate receptors.

Nuanualsuwan & Cliver (2002) exploited the ET-RTPCR approach described above in conjunction with plaque assays, cellular binding and antibody binding studies to characterise capsid behaviour during the inactivation of PV, HAV, (*Picornaviridae*) and FCV F-9 (*Caliciviridae*). The viral capsid appeared to be the primary target for UV, high temperature heat treatment (72ºC) and hypochlorite, resulting in >3 $\log_{10}$ reductions in infectivity and abolition of the RT-PCR signal following proteinase K and RNase A treatment. Viruses inactivated by low temperature heat treatment (37ºC) failed to attach to their homologous cell receptors but remained positive in RT-PCR, consistent with the formation of 135S particles with protected RNA as described above (Curry et al., 1996) Enzyme pre-treatment was not required to destroy exposed RNA for hypochlorite inactivated PV or FCV F-9, but was required for HAV, suggesting that the HAV capsid provided comparatively greater protection of the genomic RNA.

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 (faecal) samples in preparation for detection by RT-PCR. In the absence of broadly reactive antibodies, Gilpatrick et al., (2000) successfully demonstrated that human hyper-immune serum could be used for the capture of these viruses. However, such approaches are complicated by non-specific binding and a lack of common shared antigenic epitopes for the detection of all hNoVs. Immunocapture RT-PCR

approaches have been reviewed elsewhere (Rodriguez et al., 2009). Nonetheless, it has been argued that immunomagnetic separation promotes the recovery of intact infectious virus, and 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.

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 glycopeptide moieties associated with the human histo-blood group antigens (HBGAs), synthetic HBGAs have been used as capture ligands (Cannon et al., 2008; Morton et al., 2009). Other potential ligands might include peptides and nucleic acid aptamers, both of which are in various stages of development.

More recently Li et al., (2011) have extended this approach using MNV-1 and dilute faecal samples containing hNoVs. By preceding amplification with ligand binding, these investigators showed that after a treatment of 70ºC for 3 minutes (corresponding to a >5 $\log_{10}$ reduction in MNV-1 infectivity) there was a small decrease (1 $\log_{10}$) in virus titre using the cell binding RT-qPCR compared with conventional RT-qPCR. RT-qPCR signals for hNoVs were relatively unaffected by heating to 70ºC for 3 minutes but showed reduced binding (1-2 $\log_{10}$) for Caco-2 cells and pig gastric mucin. Taken together, it appears that integrated approaches are useful for measuring the loss of infectivity of MNV-1, other viruses, and potentially also for human hNoVs in conjunction with appropriate receptors. However, exactly which receptors, binding conditions, or cell lines are appropriate for broadly reactive assays remains to be determined.

7.2.4 Measurement of capsid integrity using RT-qPCR and intercalators

Pre-treatment with nucleic acid intercalating agents such as ethidium monoazide (EMA) and propidium monoazide (PMA) have been proposed as a means by which to discriminate between infectious and non-infectious virus particles. Theoretically, these compounds cannot penetrate intact capsids but are able to penetrate damaged or destroyed capsids. Once penetrated, the photo-inducible azide group on these molecules covalently cross-links the RNA, producing stable monoadducts

that cannot be amplified by PCR. This approach was reportedly able to eliminate RT-qPCR detection when applied to virus suspensions (PV, echovirus 7, and coxsackievirus B5) that were inactivated by heat (72ºC and 37ºC) or hypochlorite (Parshionikar et al., 2010). More recently, Sanchez et al., (2012) demonstrated that PMA pre-treatment of thermally inactivated HAV was more effective that RNase pre-treatment for differentiating infectious from non-infectious viruses using RT-PCR.

Nonetheless, this method is not without its drawbacks. Nucleic acid intercalating agents require double stranded regions, while most enteric viruses have genomes consisting of single-stranded RNA. Theoretically, the RT-PR assay can be designed to target an area of the genome having extensive secondary structure, and hence more likely to interact with PMA, yet such regions are frequently less amenable to efficient amplification owing to that secondary structure. Further, finding highly conserved regions of the hNoV genome is challenging, let alone restricting choices based on the need for extensive secondary structure. Further, studies to evaluate this approach using the bacteriophage T4 have suggested the method is relatively unreliable for differentiating infectious particles (Fittipaldi et al., 2010). At the current time, it is undecided as to whether nucleic acid intercalating agents can be used to measure hNoV capsid integrity.

8.0 Differentiation of RT-qPCR signals from intact and degraded virus particles

Based on this review, several RT-qPCR signal “classifications” associated with loss of capsid integrity and infectivity can be identified using the most well studied enteric RNA virus, PV. These signals can be differentiated by their susceptibility to heat treatment and resistance to RNase digestion (Table 2). In addition 160S particles may be rendered inactive by internal enzymatic genomic degradation or single strand breaks (as above) and this may not be distinguishable by RT-qPCR. Whether or not these have hNoV equivalents is unknown, however, they provide some useful examples for further discussion.

Table 2: Differentiation of different states of infectious and heat inactivated poliovirus according to infectivity, capsid integrity, receptor binding and RNase sensitivity.

Poliovirus state	Infectivity	Capsid integrity	RNA integrity	Receptor binding	Digestion of RNA by RNase	Digestion of RNA by RNase following heat treatment*
160S infectious virus	Yes	Yes	Yes	Yes	No	Yes
135S particle	+/-	+/-	Yes	+/-	No	Yes
80S.e	No	+/-	Yes	No	+/-	+/-
80S.I	No	+/-	Yes	No	Yes	Yes
Genomic RNA	+/-	No	Yes	N/A	Yes	Yes
RNPs	No	No	Yes	No	No	No
Degraded RNA	No	No	No	N/A	Yes	Yes

*Heat treatment equivalent to heating at 72 ºC for 2 minutes. +/- Intermediate or reduced response

N/A Not applicable.

80S equivalent empty capsid shells are frequently observed by EM in faecal suspensions obtained from hNoV-infected humans. Ribonucleoproteins (RNPs) result from the dissociation of capsid protein and RNA following heat treatment and their subsequent re-association to form RNase resistant RNP strands (McGregor & Mayor, 1971, McGregor, 1970, McGregor & Mayor, 1968). Heat inactivation experiments for PV have shown that even under mild inactivation conditions (45ºC for 1.5min), residual RNase resistant RNA can be released from virus particles (McGregor & Mayor, 1971). RNPs are evident in other viruses (Nuanualsuwan & Cliver, 2002) and residual RNase resistant RT-qPCR signals are obtained from hNoVs and hepatitis E following heat treatment (Schielke et al., 2011; Topping et al., 2009). The occurrence of RNPs in the environment is unknown.

The RNA within Infectious particles is naturally RNase resistant, so RNase treatment should be able to distinguish intact particles from degraded RNA. RNase activity is very stable and ubiquitous in the environment. Although high molecular weight intact virus genomic RNA is unlikely to persist in the environment in the absence of a functional capsid small low molecular weight RNA fragments or RNPs may remain detectable. Detection of these sequences may result in false positive signals in association with environmental and food samples. Since naked RNA does not

bioaccumulate significantly in shellfish (Dancer et al., 2010), this particular sample type may not be at risk for false positive results.

The NoV capsid as present in clinical samples appears particularly resilient to environmental stress. Data has shown that RNase resistant and therefore presumed intact and infectious NoVs can persist and remain infectious for human volunteers following at least 61 days storage in groundwater, (the last measured time point) and signals remained detectable for 1,266 days (Seitz et al., 2011). RNase treatment has also been used to distinguish RT-qPCR signals obtained by different virus purification methods reliant on charge-based separation or virus particle capture (Tian et al., 2012) allowing the differentiation of signals from environmental RNA and intact virus particles.

The capsid of hNoVs and hNoV VLPs are less stable at high pH (Ausar et al., 2006; Cuellar et al., 2010; Nowak et al., 2011b; Shoemaker et al., 2010) and it is not clear at present if the proposed CEN methods that use high pH steps result in the dissociation of RNA from virus particles. This is important when considering if RNase digestion steps can be applied in conjunction with CEN methods in order to discriminate naked RNA from intact particles and RNPs. On the other hand, MNV-1 retains infectivity at pH 10 (Cannon et al., 2006), and it is possible that the hNoV capsid behaves similarly. Further research is required in order to differentiate the origin of RT-qPCR signals relative to infective and inactivated virus particles.

9.0 Horizon scanning

Different approaches to detect human enteric viruses have been recently reviewed (Hamza et al., 2011). It is anticipated that continued efforts will be made to culture hNoVs. The advantages of cell culture infectivity models are that they both model infection and require active virus replication. Many viruses do not grow well in culture and require passaging and adaption to improve their growth characteristics. Human NoV RNA can be transfected into cells replicate and produce particles in cell culture although the particles are not infective (Asanaka et al., 2005). Current

strategies for developing infectivity assays involve methods designed to measure the early stages of infection through the use of indicator cell lines. These approaches currently rely on either the detection of virus expressed protein e.g. viral proteases (Cantera et al., 2010) or reporting of viral replication using transfected reporter probes (Ganguli et al., 2011). Such approaches can result in sensitive and rapid detection of replication competent infective particles in as little as 12hrs. Although this represents a significant advance on traditional virus culture, the immediate application of these approaches to hNoVs is limited. Unlike model viruses, hNoV replication does not result in infective particles capable of infecting neighbouring cells and therefore the current methods are likely to be insensitive and not applicable to environmental and food samples.

Human NoVs are diverse and there is no certainty that any developed culture model will actually prove useful as a detection system since the numbers of particles present in environmental samples is likely to be low, with possibly a high multiplicity of infection. hNoVs are considered highly infectious in susceptible human hosts and therefore there will be continuing demand to utilise the most sensitive detection methods. Continued research will investigate the relationship between PCR signals and infectivity supported by large scale genomic studies investigating the distribution of full length NoV sequences in the environment. Alternative measures of capsid integrity include the measurement of oxidative damage to the virus capsid however this unlikely to be sufficiently sensitive to determining integrity of capsid proteins in food and environmental samples (Sano et al., 2010). Additionally there are numerous analytical techniques potentially capable of single molecule detection however such methods generally involve expensive analytical equipment and are always reliant on efficient separation concentration techniques. However the applications of RT-qPCR assays in food testing laboratories will be facilitated by commercially available separation and concentration systems that will facilitate larger scale sampling making the application of RT-qPCR assays for hNoVs more routine.

10.0 Concluding Remarks

Comparison of data in this review was complicated by the wide range of different viruses and experimental conditions used in different studies. In particular experimental conditions need to be carefully controlled to allow comparison of studies. Particular attention should be paid to important variables including treatment type and conditions, virus concentration, matrix composition, enzyme activity, and potential RT-PCR inhibition. Nevertheless, the review has identified clear gaps in our knowledge regarding the detection of infectious hNoVs and these are listed below:

- There remains an absence of a suitable culture system for hNoVs.
- Defining an “infectious” virus is complicated and there is no standard as to what constitutes infectious hNoV. However, it is clear that RT-qPCR methods alone cannot distinguish infective and non-infectious virus.
- Current RT-qPCR methods for the detection of hNoVs rely on the generation and detection of a fluorescent signal without secondary confirmation of product identity. Improved characterisation of RT-qPCR products by direct sequencing or electrophoresis would increase confidence in the validity of current test results.
- Viral RNA and RNPs may persist in the environment leading to false positive identification of infectious hNoVs. The occurrence of RNPs in the environment is not known. However it is known that hNoVs are resilient and can persist and retain infectivity in the environment.
- RT-qPCR signals may be obtained from intact virus particles, and the products of degraded virus particles, ie virus RNA and ribonucleoprotein complexes (RNPs). Sample pre-treatment can allow differentiation of these RT-qPCR signals. More research is required in order to differentiate these signals reliably in relation to infectious particles and investigate their application to the proposed CEN methods. The application of “long” RT-qPCR should also be considered as a measure of genomic integrity.

Current detection methods cannot distinguish environmental sources of hNoV RNA from intact virus particles that may be presumed infectious. Data from a wide range of viruses has shown that significant exposure of capsid protected RNA occurs following heat treatment and that capsid RNA exposure assays can differentiate intact virus particles from empty capsids and free RNA. This approach could be used in food testing laboratories. However a clear gap in our knowledge exists regarding the application of such assays to the proposed CEN methods since the impact of the extraction procedures on capsid integrity has not been evaluated. Additionally long RT-qPCR assays may be used to differentiate intact from degraded genomic RNA and provide additional information on the survival of genomic RNA following different NoV treatments and processes. However, it is not known if this approach is sufficiently sensitive for characterisation of hNoVs in foods and the environment.

Finally from an analytical perspective RT-qPCR approaches for both hNoV detection and measurement need to be based on international agreement of the definition of a positive result.

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12.0 References

Reference List

Asanaka, M., R. L. Atmar, V. Ruvolo, S. E. Crawford, F. H. Neill, and M. K. Estes. 2005. Replication and packaging of Norwalk virus RNA in cultured mammalian cells. Proc. Natl. Acad. Sci. U. S. A 102(29):10327-10332.

Ausar, S. F., T. R. Foubert, M. H. Hudson, T. S. Vedvick, and C. R. Middaugh. 2006. Conformational stability and disassembly of Norwalk virus-like particles. Effect of pH and temperature. J. Biol. Chem. 281(28):19478-19488.

Bachrach, H. L. 1965. Foot-and-Mouth disease virus structural changes during reaction with cations and formaldehyde as deduced from absorbance measurements. Virology 25:532-540.

Bachrach, H.L. 1959. Foot-and-mouth disease virus: stability of its ribonucleic acid core to acid and heat. *Biochem Biophys Res Comm.* **1:**356-360

Baert, L., K. Mattison, F. Loisy-Hamon, J. Harlow, A. Martyres, B. Lebeau, A. Stals, C. E. Van, L. Herman, and M. Uyttendaele. 2011. Review: norovirus prevalence in Belgian, Canadian and French fresh produce: a threat to human health? Int. J. Food Microbiol. 151(3):261-269.

Baert, L., C. E. Wobus, C. E. Van, 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(2):543-546.

Bertolotti-Ciarlet, A., S. E. Crawford, A. M. Hutson, and M. K. Estes. 2003. The 3' end of Norwalk virus mRNA contains determinants that regulate the expression and stability of the viral capsid protein VP1: a novel function for the VP2 protein. J. Virol. 77(21):11603-11615.

Boom, R., C. J. Sol, M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen, and J. van der Noordaa. 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. 28(3):495-503.

Bostina, M., H. Levy, D. J. Filman, and J. M. Hogle. 2011. Poliovirus RNA is released from the capsid near a twofold symmetry axis. J. Virol. 85(2):776-783.

Boxman, I. L., L. Verhoef, R. Dijkman, G. Hagele, N. A. Te Loeke, and M. Koopmans. 2011. Year-round prevalence of norovirus in the environment of catering companies without a recently reported outbreak of gastroenteritis. Appl. Environ. Microbiol. 77(9):2968-2974.

Brandenburg, B., L. Y. Lee, M. Lakadamyali, M. J. Rust, X. Zhuang, and J. M. Hogle. 2007. Imaging poliovirus entry in live cells. PLoS. Biol. 5(7):e183.

Breindl, M. 1971. The structure of heated poliovirus particles. J. Gen. Virol. 11(3):147-156.

Bruggink, L. D., K. J. Witlox, R. Sameer, M. G. Catton, and J. A. Marshall. 2011. Evaluation of the RIDA((R))QUICK immunochromatographic norovirus detection assay using specimens from Australian gastroenteritis incidents. J. Virol. Methods 173(1):121-126.

Burns, M., and Valdivia, H. 2007. Modelling the limit of detection in real-time quantitative PCR .Eur Food Res Technol. 226:1513-1524

Burton-MacLeod, J. A., E. M. Kane, R. S. Beard, L. A. Hadley, R. I. Glass, and T. Ando. 2004. Evaluation and comparison of two commercial enzyme-linked immunosorbent assay kits for detection of antigenically diverse human noroviruses in stool samples. J. Clin. Microbiol. 42(6):2587-2595.

Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele, and C. T. Wittwer. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55(4):611-622.

Cannon,J.L., and Vinje,J. (2008) Histo-blood group antigen assay for detecting noroviruses in water. *Appl Environ Microbiol* **74**: 6818-6819.

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(11):2761-2765.

Cantera, J. L., W. Chen, and M. V. Yates. 2010. Detection of infective poliovirus by a simple, rapid, and sensitive flow cytometry method based on fluorescence resonance energy transfer technology. Appl. Environ. Microbiol. 76(2):584-588.

Chen, R., J. D. Neill, M. K. Estes, and B. V. Prasad. 2006. X-ray structure of a native calicivirus: structural insights into antigenic diversity and host specificity. Proc. Natl. Acad. Sci. U. S. A 103(21):8048-8053.

Cliver, D. O. 2009. Capsid and Infectivity in Virus Detection. Food Environ. Virol. 1(3-4):123-128.

Costantini, V., L. Grenz, A. Fritzinger, D. Lewis, C. Biggs, A. Hale, and J. Vinje. 2010. Diagnostic accuracy and analytical sensitivity of IDEIA Norovirus assay for routine screening of human norovirus. J. Clin. Microbiol. 48(8):2770-2778.

Cuellar, J. L., F. Meinhoevel, M. Hoehne, and E. Donath. 2010. Size and mechanical stability of norovirus capsids depend on pH: a nanoindentation study. J. Gen. Virol. 91(Pt 10):2449-2456.

Curry, S., M. Chow, and J. M. Hogle. 1996. The poliovirus 135S particle is infectious. J. Virol. 70(10):7125-7131.

Dancer, D., R. E. Rangdale, J. A. Lowther, and D. N. Lees. 2010. Human norovirus RNA persists in seawater under simulated winter conditions but does not bioaccumulate efficiently in Pacific Oysters (Crassostrea gigas). J. Food Prot. 73(11):2123-2127.

De Roda Husman, A. M., W. J. Lodder, S. A. Rutjes, J. F. Schijven, and P. F. Teunis. 2009. Long-term inactivation study of three enteroviruses in artificial surface and groundwaters, using PCR and cell culture. Appl. Environ. Microbiol. 75(4):1050-1057.

De Bruin E., E. Duizer, H. Vennema, and M. P. Koopmans. 2006. Diagnosis of Norovirus outbreaks by commercial ELISA or RT-PCR. J. Virol. Methods 137(2):259-264.

Denoya, C. D., E. A. Scodeller, C. Vasquez, and J. L. La Torre. 1978a. Foot and mouth disease virus. II. Endoribonuclease activity within purified virions. Virology 89(1):67-74.

Denoya, C. D., E. A. Scodeller, C. Vasquez, and J. L. La Torre. 1978b. Ribonuclease activities associated with purified foot and mouth disease virus. Arch. Virol. 57(2):153-159.

Diez-Valcarce, M., Kovac, K., Raspor, P., Rodriguez-Lazaro, D., Hernandez, M. (2011) Virus genome quantification does not predict norovirus infectivity after application of food inactivation processing technologies. Food Environ Virol 3:141-146

Dimmock, N. J. 1967. Differences between the thermal inactivation of picornaviruses at "high" and "low" temperatures. Virology 31(2):338-353.

Doel, T. R., and P. J. Baccarini. 1981. Thermal stability of foot-and-mouth disease virus. Arch. Virol. 70(1):21-32.

Duizer, E., K. J. Schwab, F. H. Neill, R. L. Atmar, M. P. Koopmans, and M. K. Estes. 2004a. Laboratory efforts to cultivate noroviruses. J. Gen. Virol. 85(Pt 1):79-87.

Duizer, E., P. Bijkerk, B. Rockx, G. A. De, F. Twisk, and M. Koopmans. 2004b. Inactivation of caliciviruses. Appl. Environ. Microbiol. 70(8):4538-4543.

Fisman, D. N., A. L. Greer, G. Brouhanski, and S. J. Drews. 2009. Of gastro and the gold standard: evaluation and policy implications of norovirus test performance for outbreak detection. J. Transl. Med. 7:23.

Fittipaldi, M., N. J. Rodriguez, F. Codony, B. Adrados, G. A. Penuela, and J. Morato. 2010. Discrimination of infectious bacteriophage T4 virus by propidium monoazide real-time PCR. J. Virol. Methods 168(1-2):228-232.

Fukuda, S., Y. Sasaki, and M. Seno. 2008. Rapid and sensitive detection of norovirus genomes in oysters by a two-step isothermal amplification assay system combining nucleic acid sequence-based amplification and reverse transcription-loop-mediated isothermal amplification assays. Appl. Environ. Microbiol. 74(12):3912-3914.

Fukuda, S., S. Takao, M. Kuwayama, Y. Shimazu, and K. Miyazaki. 2006. Rapid detection of norovirus from fecal specimens by real-time reverse transcription-loop-mediated isothermal amplification assay. J. Clin. Microbiol. 44(4):1376-1381.

Ganguli, P. S., W. Chen, and M. V. Yates. 2011. Detection of murine norovirus-1 by using TAT peptide-delivered molecular beacons. Appl. Environ. Microbiol. 77(15):5517-5520.

Gerondopoulos, A., T. Jackson, P. Monaghan, N. Doyle, and L. O. Roberts. 2010. Murine norovirus-1 cell entry is mediated through a non-clathrin-, non-caveolae-, dynamin- and cholesterol-dependent pathway. J. Gen. Virol. 91(Pt 6):1428-1438.

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

Groppelli, E., T. J. Tuthill, and D. J. Rowlands. 2010. Cell entry of the aphthovirus equine rhinitis A virus is dependent on endosome acidification. J. Virol. 84(12):6235-6240.

Hamza, I. A., L. Jurzik, K. Uberla, and M. Wilhelm. 2011. Methods to detect infectious human enteric viruses in environmental water samples. Int. J. Hyg. Environ. Health 214(6):424-436.

Hardy, M. E., L. J. White, J. M. Ball, and M. K. Estes. 1995. Specific proteolytic cleavage of recombinant Norwalk virus capsid protein. J. Virol. 69(3):1693-1698.

Hartnell, R., J. Lowther, J. Avant, D. Dancer, D. Lees, and J. Russell. 2012. The development of LENTICULES as reference materials for noroviruses. J. Appl. Microbiol. 112(2):338-345.

Herrmann, J. E., and D. O. Cliver. 1973. Degradation of coxsackievirus type A9 by proteolytic enzymes. Infect. Immun. 7(4):513-517.

Hiatt, C. W. 1964. Kinetics of the inactivation of viruses. Bacteriol. Rev. 28:150-163.

Holland, J. J., B. H. Hoyer, L. C. McLaren, and J. T. Syverton. 1960. Enteroviral ribonucleic acid. I. Recovery from virus and assimilation by cells. J. Exp. Med. 112:821-839.

Hot, D., O. Legeay, J. Jacques, C. Gantzer, Y. Caudrelier, K. Guyard, M. Lange, and L. Andreoletti. 2003. Detection of somatic phages, infectious enteroviruses and enterovirus genomes as indicators of human enteric viral pollution in surface water. Water Res. 37(19):4703-4710.

Houde, A., D. Leblanc, E. Poitras, P. Ward, J. Brassard, C. Simard, and Y. L. Trottier. 2006. Comparative evaluation of RT-PCR, nucleic acid sequence-based amplification (NASBA) and real-time RT-PCR for detection of noroviruses in faecal material. J. Virol. Methods 135(2):163-172.

Jaykus, L. and B. Escudero-Abarca. 2010. Human pathogenic viruses in food. In:

V.K. Juneja and J.N. Sofos (eds.) Pathogens and Toxins in Foods:

Challenges and Interventions. ASM Press, Washington, DC, pp. 218-232.

Jothikumar, N., T. L. Cromeans, M. D. Sobsey, and B. H. Robertson. 2005a. Development and evaluation of a broadly reactive TaqMan assay for rapid detection of hepatitis A virus. Appl. Environ. Microbiol. 71(6):3359-3363.

Jothikumar, N., J. A. Lowther, K. Henshilwood, D. N. Lees, V. R. Hill, and J. Vinje. 2005b. Rapid and sensitive detection of noroviruses by using TaqMan-based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples. Appl. Environ. Microbiol. 71(4):1870-1875.

Kageyama, T., S. Kojima, M. Shinohara, K. Uchida, S. Fukushi, F. B. Hoshino, N. Takeda, and K. Katayama. 2003. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. J. Clin. Microbiol. 41(4):1548-1557.

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(5):1075-1081.

Kele, B., G. Lengyel, and J. Deak. 2011. Comparison of an ELISA and two reverse transcription polymerase chain reaction methods for norovirus detection. Diagn. Microbiol. Infect. Dis. 70(4):475-478.

Kirby, A., R. Q. Gurgel, W. Dove, S. C. Vieira, N. A. Cunliffe, and L. E. Cuevas. 2010. An evaluation of the RIDASCREEN and IDEIA enzyme immunoassays

and the RIDAQUICK immunochromatographic test for the detection of norovirus in faecal specimens. J. Clin. Virol. 49(4):254-257.

Kostela, J., M. Ayers, J. Nishikawa, L. McIntyre, M. Petric, and R. Tellier. 2008. Amplification by long RT-PCR of near full-length norovirus genomes. J. Virol. Methods 149(2):226-230.

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(12):1375-1380.

Langlet,J., Gaboriaud,F., and Gantzer,C. (2007) Effects of pH on plaque forming unit counts and aggregation of MS2 bacteriophage. J Appl Microbiol 103: 1632-1638.

Larkin, E. P., and A. C. Fassolitis. 1979. Viral heat resistance and infectious ribonucleic acid. Appl. Environ. Microbiol. 38(4):650-655.

Lee, K. M., and J. H. Gillespie. 1973. Thermal and pH stability of feline calicivirus. Infect. Immun. 7(4):678-679.

Levy, H. C., M. Bostina, D. J. Filman, and J. M. Hogle. 2010. Catching a virus in the act of RNA release: a novel poliovirus uncoating intermediate characterized by cryo-electron microscopy. J. Virol. 84(9):4426-4441.

Li, D., L. Baert, C. E. Van, and M. Uyttendaele. 2011. Critical studies on binding-based RT-PCR detection of infectious Noroviruses. J. Virol. Methods 177(2):153-159.

Li, D., L. Baert, M. Xia, W. Zhong, C. E. Van, X. Jiang, and M. Uyttendaele. 2012. Evaluation of methods measuring the capsid integrity and/or functions of noroviruses by heat inactivation. J. Virol. Methods.

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(10):4951-4955.

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(6):1514-1519.

Lowther, J. A., N. E. Gustar, R. E. Hartnell, and D. N. Lees. 2012. Comparison of norovirus RNA levels in outbreak-related oysters with background environmental levels. J. Food Prot. 75(2):389-393.

Mateo, R., A. Diaz, E. Baranowski, and M. G. Mateu. 2003. Complete alanine scanning of intersubunit interfaces in a foot-and-mouth disease virus capsid

reveals critical contributions of many side chains to particle stability and viral function. J. Biol. Chem. 278(42):41019-41027.

Mattison, K., J. Harlow, V. Morton, A. Cook, F. Pollari, S. Bidawid, and J. M. Farber. 2010. Enteric viruses in ready-to-eat packaged leafy greens. Emerg. Infect. Dis. 16(11):1815-1817.

McGregor, S., and H. D. Mayor. 1968. Biophysical studies on rhinovirus and poliovirus. I. Morphology of viral ribonucleoprotein. J. Virol. 2(2):149-154

McGregor, S. 1970. Further studies of the structure of the picornavirion. Crit. Rev. Lab. Sci. 1:110-114.

McGregor, S., and H. D. Mayor. 1971. Internal components released from rhinovirus and poliovirus by heat. J. Gen. Virol. 10(2):203-207.

Mormann, S., M. Dabisch, and B. Becker. 2010. Effects of technological processes on the tenacity and inactivation of norovirus genogroup II in experimentally contaminated foods. Appl. Environ. Microbiol. 76(2):536-545.

Newman, J. F., S. Tirrell, C. Ullman, P. G. Piatti, and F. Brown. 1996. Stabilising oral poliovaccine at high ambient temperatures. Dev. Biol. Stand. 87:103-111.

Nowak, P., J. R. Topping, K. Bellamy, V. Fotheringham, J. J. Gray, J. P. Golding, G. Wiseman, and A. I. Knight. 2011a. Virolysis of feline calicivirus and human GII.4 norovirus following chlorine exposure under standardized light soil disinfection conditions. J. Food Prot. 74(12):2113-2118.

Nowak, P., J. R. Topping, V. Fotheringham, C. I. Gallimore, J. J. Gray, M. Iturriza-Gomara, and A. I. Knight. 2011b. Measurement of the virolysis of human GII.4 norovirus in response to disinfectants and sanitisers. J. Virol. Methods 174(1-2):7-11.

Nuanualsuwan, S., and D. O. Cliver. 2002. Pretreatment to avoid positive RT-PCR results with inactivated viruses. J. Virol. Methods 104(2):217-225.

Nuanualsuwan, S., and D. O. Cliver. 2003a. Capsid functions of inactivated human picornaviruses and feline calicivirus. Appl. Environ. Microbiol. 69(1):350-357.

Nuanualsuwan, S., and D. O. Cliver. 2003b. Infectivity of RNA from inactivated poliovirus. Appl. Environ. Microbiol. 69(3):1629-1632.

Park, G. W., and M. D. Sobsey. 2011. Simultaneous comparison of murine norovirus, feline calicivirus, coliphage MS2, and GII.4 norovirus to evaluate the efficacy of sodium hypochlorite against human norovirus on a fecally soiled stainless steel surface. Foodborne. Pathog. Dis. 8(9):1005-1010.

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(13):4318-4326.

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

Patterson, S. S., M. W. Smith, E. T. Casper, D. Huffman, L. Stark, D. Fries, and J. H. Paul. 2006. A nucleic acid sequence-based amplification assay for real-time detection of norovirus genogroup II. J. Appl. Microbiol. 101(4):956-963.

Pecson, B. M., L. V. Martin, and T. Kohn. 2009. Quantitative PCR for determining the infectivity of bacteriophage MS2 upon inactivation by heat, UV-B radiation, and singlet oxygen: advantages and limitations of an enzymatic treatment to reduce false-positive results. Appl. Environ. Microbiol. 75(17):5544-5554.

Richards, G. P. 1999. Limitations of molecular biological techniques for assessing the virological safety of foods. J. Food Prot. 62(6):691-697.

Rodriguez, R. A., I. L. Pepper, and C. P. Gerba. 2009. Application of PCR-based methods to assess the infectivity of enteric viruses in environmental samples. Appl. Environ. Microbiol. 75(2):297-307.

Ros, C., C. Baltzer, B. Mani, and C. Kempf. 2006. Parvovirus uncoating in vitro reveals a mechanism of DNA release without capsid disassembly and striking differences in encapsidated DNA stability. Virology 345(1):137-147.

Sair, A. I., D. H. D'Souza, C. L. Moe, and L. A. Jaykus. 2002. Improved detection of human enteric viruses in foods by RT-PCR. J. Virol. Methods 100(1-2):57-69.

Salo, R. J., and D. O. Cliver. 1976. Effect of acid pH, salts, and temperature on the infectivity and physical integrity of enteroviruses. Arch. Virol. 52(4):269-282.

Sano, D., R. M. Pinto, T. Omura, and A. Bosch. 2010. Detection of oxidative damages on viral capsid protein for evaluating structural integrity and infectivity of human norovirus. Environ. Sci. Technol. 44(2):808-812.

Schielke, A., M. Filter, B. Appel, and R. Johne. 2011. Thermal stability of hepatitis E virus assessed by a molecular biological approach. Virol. J. 8:487.

Schwab, K. J., F. H. Neill, G. F. Le, M. K. Estes, and R. L. Atmar. 2001. Development of a reverse transcription-PCR-DNA enzyme immunoassay for detection of "Norwalk-like" viruses and hepatitis A virus in stool and shellfish. Appl. Environ. Microbiol. 67(2):742-749.

Scodeller, E. A., M. A. Lebendiker, M. S. Dubra, O. A. Crespo, O. Basarab, J. L. La Torre, and C. Vasquez. 1984. Inactivation of foot-and-mouth disease virus

vaccine strains by activation of virus-associated endonuclease. J. Gen. Virol. 65 (Pt 9):1567-1573.

Seitz,S.R., Leon,J.S., Schwab,K.J., Lyon,G.M., Dowd,M., McDaniels,M. et al. 2011 Norovirus infectivity in humans and persistence in water. Appl Environ Microbiol 77: 6884-6888.

Shoemaker, G. K., D. E. van, S. E. Crawford, C. Uetrecht, M. Baclayon, W. H. Roos, G. J. Wuite, M. K. Estes, B. V. Prasad, and A. J. Heck. 2010. Norwalk virus assembly and stability monitored by mass spectrometry. Mol. Cell Proteomics. 9(8):1742-1751.

Siebenga, J. J., H. Vennema, D. P. Zheng, J. Vinje, B. E. Lee, X. L. Pang, E. C. Ho, W. Lim, A. Choudekar, S. Broor, T. Halperin, N. B. Rasool, J. Hewitt, G. E. Greening, M. Jin, Z. J. Duan, Y. Lucero, M. O'Ryan, M. Hoehne, E. Schreier, R. M. Ratcliff, P. A. White, N. Iritani, G. Reuter, and M. Koopmans. 2009. Norovirus illness is a global problem: emergence and spread of norovirus GII.4 variants, 2001-2007. J. Infect. Dis. 200(5):802-812.

Siegl, G., M. Weitz, and G. Kronauer. 1984. Stability of hepatitis A virus. Intervirology 22(4):218-226.

Simonet, J., and C. Gantzer. 2006. Degradation of the Poliovirus 1 genome by chlorine dioxide. J. Appl. Microbiol. 100(4):862-870.

Stals,A., Baert,L., Van,C.E., and Uyttendaele,M. (2012) Extraction of food-borne viruses from food samples: A review. *Int J Food Microbiol* **153**: 1-9.

Stals, A., L. Baert, V. Jasson, C. E. Van, and M. Uyttendaele. 2011. Screening of fruit products for norovirus and the difficulty of interpreting positive PCR results. J. Food Prot. 74(3):425-431.

Stals, A., H. Werbrouck, L. Baert, N. Botteldoorn, L. Herman, M. Uyttendaele, and C. E. Van. 2009. Laboratory efforts to eliminate contamination problems in the real-time RT-PCR detection of noroviruses. J. Microbiol. Methods 77(1):72-76.

Straub, T. M., R. A. Bartholomew, C. O. Valdez, N. B. Valentine, A. Dohnalkova, R. M. Ozanich, C. J. Bruckner-Lea, and D. R. Call. 2011. Human norovirus infection of caco-2 cells grown as a three-dimensional tissue structure. J. Water Health 9(2):225-240.

Stuart, A. D., and T. D. Brown. 2006. Entry of feline calicivirus is dependent on clathrin-mediated endocytosis and acidification in endosomes. J. Virol. 80(15):7500-7509.

Terry, G. M., A. Syred, and M. M. Rweyemamu. 1983. Studies on the stability of foot-and-mouth disease virus using absorbance - temperature profiles. Dev. Biol. Stand. 55:117-120.

Teunis, P. F., C. L. Moe, P. Liu, S. E. Miller, L. Lindesmith, R. S. Baric, P. J. Le, and R. L. Calderon. 2008. Norwalk virus: how infectious is it? J. Med. Virol. 80(8):1468-1476.

Tian, P., A. Engelbrektson, and R. Mandrell. 2008. Two-log increase in sensitivity for detection of norovirus in complex samples by concentration with porcine gastric mucin conjugated to magnetic beads. Appl. Environ. Microbiol. 74(14):4271-4276.

Tian, P., D. Yang, X. Jiang, W. Zhong, J. L. Cannon, W. Burkhardt, III, J. W. Woods, G. Hartman, L. Lindesmith, R. S. Baric, and R. Mandrell. 2010. Specificity and kinetics of norovirus binding to magnetic bead-conjugated histo-blood group antigens. J. Appl. Microbiol. 109(5):1753-1762.

Tian, P., D. Yang, and R. Mandrell. 2011. A simple method to recover Norovirus from fresh produce with large sample size by using histo-blood group antigen-conjugated to magnetic beads in a recirculating affinity magnetic separation system (RCAMS). Int. J. Food Microbiol. 147(3):223-227.

Tian, P., D. Yang, L. Pan, and R. Mandrell. 2012. Application of a Receptor-Binding Capture Quantitative Reverse Transcription-PCR Assay To Concentrate Human Norovirus from Sewage and To Study the Distribution and Stability of the Virus. Appl. Environ. Microbiol. 78(2):429-436.

Topping, J. R., H. Schnerr, J. Haines, M. Scott, M. J. Carter, M. M. Willcocks, K. Bellamy, D. W. Brown, J. J. Gray, C. I. Gallimore, and A. I. Knight. 2009. Temperature inactivation of Feline calicivirus vaccine strain FCV F-9 in comparison with human noroviruses using an RNA exposure assay and reverse transcribed quantitative real-time polymerase chain reaction-A novel method for predicting virus infectivity. J. Virol. Methods 156(1-2):89-95.

Wetz, K., and T. Kucinski. 1991. Influence of different ionic and pH environments on structural alterations of poliovirus and their possible relation to virus uncoating. J. Gen. Virol. 72 (Pt 10):2541-2544.

Wiegers, U., and H. Hilz. 1971. A new method using 'proteinase K' to prevent mRNA degradation during isolation from HeLa cells. Biochem. Biophys. Res. Commun. 44(2):513-519.

Wolf, S., Rivera-Aban, M., Greening, G.G. 2009. Long-range reverse transcription as a useful tool to assess the genomic integrity of norovirus. Food. Environ. Virol 1:129-136.

13.0 Appendix

The review was built upon three recent reviews (Cliver, 2009; Hamza et al., 2011; Rodriguez et al., 2009) relevant to the objectives of this proposal.

Additionally the search terms below were used to research the literature in order to provide a comprehensive literature review relevant to the objectives.

Search terms included: Capsid, stability, integrity, infectivity, inactivation, infectious , assembly, disassembly, dissociation, destruction, receptor binding, antibody binding, cell capture, cell attachment RT-qPCR or RT-PCR, RNase, virolysis, ribonuclease, pretreatment, RT-qPCR, proteinase K, propium monoazide, intercalator, cell capture, long, RT-qPCR, discrimination, differentiation, infectivity, infectious, novel, method, measurement, assay, virus.

Additionally the authors contributed personal expertise in virus infectivity resulting in a comprehensive literature review of capsid stability and its relationship with virus infectivity spanning fifty years of published data and citing over 100 references.