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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

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A B S T R A C T

A one-step reverse transcription quantitative real-time polymerase chain reaction (RT-QPCR) method in combination with RNase treatment and low copy number samples was developed in order to examine the effect of temperature on the ability of virus capsids to protect their RNA content. The method was applied to a non-cultivable virus (GII.4 norovirus) and Feline calicivirus vaccine strain F-9 (FCV) which is often used as a norovirus surrogate. Results demonstrated that FCV RNA is exposed maximally after 2 min at 63.3 °C and this correlated with a greater than 4.5 log reduction in infectivity as assessed by plaque assay. In contrast human GII.4 norovirus RNA present in diluted clinical specimens was not exposed maximally until 76.6 °C, at least 13.3 °C greater than that for FCV. These data suggest that norovirus possesses greater thermostability than this commonly used surrogate. Further, these studies indicate that current food processing regimes for pasteurisation are insufficient to achieve inactivation of GII.4 NoVs. The method provides a novel molecular method for predicting virus infectivity.

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1. Introduction

The non-cultivable noroviruses (NoVs) have emerged as the single most common cause of acute non-bacterial gastroenteritis in the industrialised world. Estimates of foodborne transmission vary widely between countries with food and water associated with 7–24% of all outbreaks (Lopman et al., 2003). Trends towards the increasing use of prepared foods (particularly, those consumed without further cooking), consumption of fresh produce and fresh produce containing foods, consumption of raw seafood and eating “out of home” may all have contributed to this estimate, although improved surveillance and reporting will also have increased awareness of foodborne gastroenteritis. Food produce may be contaminated with viruses at source or at any stage during processing, packaging and distribution. NoVs are transmitted through the faecal–oral route, by consumption of faecally contaminated food or water (Seymour and Appleton, 2001; Wyn-Jones and Sellwood, 2001) or by direct person-to-person spread. NoVs are highly contagious, and as few as 10 viral particles may be sufficient to cause infection (Caul, 1994).

Although comparatively recent reports suggest that it may now be possible to culture NoVs in a complex culture system (Straub et al., 2007) the utility of this system remains to be confirmed. The lack of a simple culture system for the biological assay of NoVs has hindered the development and verification of established control measures that may be used in hazard analysis and critical control point (HACCP) systems and pre-requisite programmes to control NoVs in food processing and preparation environments. Although a number of studies have used Feline calicivirus [FCV] (Bidawid et al., 2004; Doultree et al., 1999; Duizer et al., 2004; Gulati et al., 2001; Lopman et al., 2002; Slomka and Appleton, 1998) and more recently the newly described Murine norovirus [MNV-1] (Wobus et al., 2004) as surrogates to monitor virus survival (Cannon et al.,

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2006); it remains to be shown whether either of these two viruses reflect accurately the behaviour of NoVs in food processing.

Current means for the detection and monitoring of norovirus contamination/survival rely heavily on physical methods of detection, mainly RT-PCR. However, this approach cannot inform as to whether the signals detected represent infectious virus that poses a threat, or remnants from inactivated virus that are harmless. The aim of this investigation was to develop a molecular approach towards predicting viral infectivity by coupling PCR detection to other properties of an infective virus: in this case the presence of an intact capsid. One measure of the integrity of a virus capsid is its ability to protect the genomic RNA within. We have therefore combined quantitative real-time RT-PCR with an RNase protection assay that can provide an indication of capsid damage in response to various stresses, in this case heat treatment.

Previous attempts to develop such a technique have shown that reductions in viral RNA copy number are related broadly to reductions in infectivity only under extreme conditions of inactivation (Duizer et al., 2004). There was no clear correlation under relatively mild inactivation conditions leading to the conclusion that in these cases RNA might continue to experience some degree of residual protection (Nuanualsuwan and Cliver, 2003; Baert et al., 2008). This has meant that RNase protection methods have hitherto offered no advantage over direct RT-QPCR and prevented their widespread adoption in the study of virus response to more mild insults (Baert et al., 2008).

The apparent disconnection between RNA destruction and infectivity under mild conditions may be a product of the methods employed and a consequence of residual RNA protection: most determinations have used high copy number samples and have sought large reductions in virus RNA. Under these circumstances apparently small reductions caused by relatively mild conditions lead to only a very small reduction in threshold cycle (Ct) value. Although such differences may appear insignificant, they actually correspond to large differences in copy number because of the exponential nature of the PCR. These effects can be detected more sensitively at low input levels of virus. Therefore in order to examine these differences in more detail we have performed our studies using lower copy number samples and a less specific RNase, RNase ONE. This can expand the Ct response range available for analysis compared with high copy number samples. Although absolute PCR quantitation at low copy number is difficult this problem by can be mitigated by using adequate comparators within the experiment itself, i.e. measurement of relative copy number. This eliminates the need for absolute copy number determination and normalises the efficiency of the RT and PCR steps between all samples. The low copy number RNase-protection approach was used to characterise the thermal inactivation of FCV strain F-9 in terms of loss of capsid integrity (RNA exposure) and also by conventional plaque assay. In addition these data have been compared with RNA exposure data derived from GII.4 NoVs in order to assess the thermal resistance of GII.4 NoVs capsids in comparison to that of the surrogate FCV F-9.

2. Materials and methods

2.1. Virus samples and plaque assays

Feline calicivirus FCV F-9 culture conditions and plaque assays were as described by Cannon et al. (2006). Stock FCV F-9 was kindly supplied by M.J.C. and diluted in Dulbecco's phosphate buffered saline (PBS) pH 7.0 for inactivation studies and plaque assays. GII.4 positive stool samples were resuspended at 10% (wt/vol) in PBS, briefly clarified by micro-centrifugation, and diluted 1:100–1:1000 in PBS for use in inactivation studies. Three different epidemiologically unlinked GII.4 norovirus clinical isolates were examined and obtained originally from outbreaks aboard cruise ships. Isolates were kindly supplied by the UK Health Protection Agency (H07260249, H071400432 and H072020533. H072020533 (NoV(i)) and H071400432 (NoV(ii)) were used for FCV F-9 spiking experiments.

2.2. Thermal inactivation

For heat inactivation, volumes (50–100 μl) of virus dilution containing 10^4 to 10^5 RT-QPCR copies were subjected to heat inactivation for 2 min at different temperatures in thin walled PCR tubes in a thermal cycler.

2.3. RNase treatment

Following thermal treatment experimental samples were adjusted to 100 μl with PBS. RNase treatment was carried out by the addition of 11 μl 10× reaction buffer and 1 μl (10 units) RNase ONE™ Ribonuclease (Promega UK Ltd., Southampton, UK). The solution was incubated at 37 °C for 15 min. Following RNase treatment the volume was further adjusted to 140 μl with PBS prior to RNA isolation. Control samples containing RNase buffer but without RNase were kept on ice.

2.4. RNA isolation

Viral RNA was extracted using a QIAamp viral RNA kit (Qiagen Ltd., Crawley, UK) according to the manufacturer's instructions. RNA was eluted with 60 μl of AVE-buffer provided and 2 μl used directly in RT-QPCR.

2.5. Reverse transcription quantitative real time PCR (RT-QPCR)

One-step RT-QPCR amplification was performed in triplicate using an iCycler iQ™ (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) and the iScript One-Step RT-PCR kit for Probes (BioRad). FCV F-9 reverse transcription was performed in a total volume of 25 μl at 50 °C for 10 min, followed by denaturation at 95 °C for 5 min, and then 45 cycles of amplification with denaturation at 95 °C for 10 s and combined annealing and extension at 60 °C for 30 s. FCV primers (15 pmol each) and TaqMan probe (6 pmol) were. *CATF1* 5′-TCT GTA TGT TGG GTG TGC CATT and *CATR1* 5′-GAT ATC AAA AGT TGA GAA AAG GTT CC and amplified an 89 bp fragment of the FCV polymerase gene. The TaqMan probe was:

FAM 5′-CCCTAGAAGCTAAGGCCATTCCCGTCAC-3′ TAMRA. NoV GII PCR, primers and TaqMan probe were as described by Kageyama et al. (2003). The PCR conditions were as for the FCV F-9 PCR described above. All primers and probes were synthesised by Applied Biosystems, UK. Amplification data were collected and analysed with iCycler iQ™ real-time detection system software version 5.0 (Bio-Rad). All amplifications were performed in triplicate.

2.6. Calculation of percentage target survival

In each experiment, an FCV F-9 or GII.4-specific standard curve was generated by a 10-fold serial dilution of cloned plasmid control template. This was used to provide an approximation of copy number and an assessment of the linearity of the log copy number and Ct values. The percentage target survival was calculated by comparison with the starting copy number. This was determined in triplicate for each experiment. No significant differences were observed in the determination of apparent copy number between RNase treated and control virus solutions demonstrating that free

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