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Discrimination of infectious and heat-treated norovirus by combining platinum compounds and real-time RT-PCR

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ABSTRACT

Human noroviruses (NoV) are major agents of foodborne outbreaks. Because of the lack of a standardized cell culture method, real-time reverse transcriptase PCR is now commonly used for the detection of NoV in foodstuffs and environmental samples. However, this approach detects the viral nucleic acids of both infectious and non-infectious viruses and needs to be optimized to predict infectivity for public health risk assessment. The aim of this study was to develop a viability PCR method to discriminate between native and heat-treated virus, for both NoV and its surrogate, murine norovirus (MNV).

To this end, screening of viability markers (monoazide dyes, platinum and palladium compounds) was performed on viral RNA, native virus or heat-treated virus, and incubation conditions were optimized with $PtCl_4$, the most efficient viability marker. Multiple MNV molecular models were designed: no impact of amplicon length was observed on inactivated MNV genomic titer; but the 5′NTR, ORF1 and 3′UTR regions resulted in higher reductions than central genomic regions. The optimal viability PCR conditions developed (incubation with 2.5 mM $PtCl_4$ in PBS for 10 min at 5 °C) were finally applied to MNV by performing heat inactivation studies and to native and heat-treated NoV clinical strains. The viability PCR discriminated efficiently between native and heat-inactivated MNV at 72 °C and 80 °C, and efficiently reduced the genomic titer of heat-treated NoV strains.

This viability PCR method could be useful to study heat inactivation kinetics of NoV and MNV. It could also be evaluated for the identification of infectious enteric viruses in foodstuffs and environmental samples.

1. Introduction

Gastroenteritis is a major public health concern and economic burden worldwide. According to the latest epidemiological data available, enteric viruses are the cause of 9 to 20% of foodborne outbreaks reported in Europe (EFSA and ECDC, 2015; EFSA and ECDC, 2016) and 48% in the United States (Hall et al., 2013). Among the enteric viruses implicated in foodborne outbreaks, human norovirus (NoV) is the leading causative agent (EFSA and ECDC, 2016; Hall et al., 2014). The economic burden of norovirus-related gastroenteritis is estimated at US $ 4.2 billion in direct health system costs and US$ 60.3 billion in societal costs per year (Bartsch et al., 2016).

NoV belongs to the *Caliciviridae* family and is a small non-enveloped virus that has a positive-sense, single-stranded RNA genome, organized into three open reading frames (ORFs). NoV is classified into six genogroups (GI–GVI), three of which (GI, GII and GIV) are responsible for human outbreaks (Kroneman et al., 2013; White, 2014). NoV is highly contagious and mainly transmitted *via* the fecal-oral route directly by person-to-person contact; or indirectly by consumption of contaminated food or water, or contact with contaminated environmental surfaces (Lopman et al., 2012; Matthews et al., 2012; Wikswo et al., 2015). Infection may induce vomiting, diarrhea, mild fever, abdominal cramping and nausea in infected children and adults. Infectious NoV particles are shed in high concentrations in the stools, enabling the virus to spread rapidly and efficiently (Sabrià et al., 2016; Teunis et al., 2015). NoV is highly stable in the environment and even a few infectious particles can induce gastroenteritis (Rzezutka and Cook, 2004; Seitz et al., 2011; Teunis et al., 2008). Therefore, it is important to have sensitive, reliable and widely applicable techniques for the detection and quantification of NoV in food, water and environmental samples.

Even though cell culture is the gold standard to examine viral infectivity, NoV detection based on infectivity is complicated by the lack of a reliable cell culture method and the low contamination levels of foodstuffs (Dicaprio et al., 2013; Moore et al., 2015). Two cell culture

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methods have recently been published for the detection of infectious NoV particles (Ettayebi et al., 2016; Jones et al., 2015) but they are not usable in routine analysis at this time.

Standard molecular methods have been developed for the rapid detection of norovirus and hepatitis A virus (HAV) in foodstuffs (ISO, 2017). They are based on viral genome detection using real-time reverse transcriptase PCR (RT-qPCR), used for its sensitivity, specificity, speed and ability to deliver quantitative data. However, this approach detects the viral nucleic acids of both infectious and non-infectious viruses (Girones et al., 2010; Knight et al., 2013; Stals et al., 2013), potentially overestimating amounts of infectious virus. In fact, the persistence of viral genome in foodstuffs and in the environment may be much greater than that of the corresponding infectious enteric virus (Baert et al., 2008; Cook et al., 2016; Seitz et al., 2011). Molecular approaches need to be optimized to predict the infectivity of contaminated samples and to assess the risk for human health (Bouwknegt et al., 2015; Van Abel et al., 2017).

Murine norovirus (MNV), an enteric pathogen of mice that belongs to the *Norovirus* genus, has frequently been used as a human norovirus surrogate to study virus stability and inactivation (Cromeans et al., 2014; Fraisse et al., 2011; Hirneisen and Kniel, 2013; Seo et al., 2012). MNV is morphologically and genetically similar to NoV, and can routinely be grown in cell culture. However, there are fundamental differences between NoV and its surrogate, such as host viral receptors, pathogenesis or cell line susceptibility (Richards, 2012; Tan and Jiang, 2010; Wobus et al., 2006). Moreover, it remains unclear whether NoV could be inactivated by the inactivation conditions established for its surrogate (Knight et al., 2016). Finally, the sensitivity to a given inactivation condition could vary considerably among NoV strains (Lou et al., 2016). Although the use of a surrogate is a promising option, direct assessment of NoV viability is essential to evaluate the infectious risk.

Besides the use of surrogates, molecular methods for the evaluation of NoV infectivity based on capsid integrity have been reported. A promising strategy is based on the use of nucleic acid intercalating dyes as a sample treatment prior to the RT-qPCR. Ethidium monoazide (EMA) and propidium monoazide (PMA) have been used for more than a decade to address the inability of conventional PCR to distinguish live bacteria from dead bacteria (Elizaquível et al., 2014; Nocker et al., 2006; Nogva et al., 2003). These viability dyes were tested on enteric viruses; they penetrate only into damaged capsids and intercalate covalently into viral nucleic acids after a photo-activation step, interfering with genome amplification by qPCR. This approach has been used successfully to discriminate between infectious and non-infectious enteroviruses (Kim et al., 2011; Leifels et al., 2015; Parshionikar et al., 2010), adenovirus (Leifels et al., 2015), rotavirus (Coudray-Meunier et al., 2013; Leifels et al., 2015), hepatitis A virus (Coudray-Meunier et al., 2013; Randazzo et al., 2018b; Sánchez et al., 2012) and NoV surrogates MNV (Kim et al., 2011; Kim and Ko, 2012; Lee et al., 2015; Leifels et al., 2015) and Tulane virus (Li et al., 2017). The approach has also been applied in foodstuffs (Jeong et al., 2017; Moreno et al., 2015; Quijada et al., 2016; Randazzo et al., 2016, 2018a, 2018b) and in environmental waters (Blanco et al., 2017; Fuster et al., 2016; Leifels et al., 2016; Parshionikar et al., 2010; Prevost et al., 2016; Randazzo et al., 2016, 2018a, 2018b). However, the efficacy of viability dyes depends on the target virus, the matrix tested, and the inactivation procedure. The application of viability dyes combined with RT-qPCR to NoV yielded mixed results for the discrimination between native and heat-treated virus: PMA and EMA were inefficient in three studies out of five and one study out of two, respectively, and PMAxx was efficient in one study (Escudero-Abarca et al., 2014; Jeong et al., 2017; Karim et al., 2015; Parshionikar et al., 2010; Randazzo et al., 2016). This lack of consistency may be due to the use of different virus strains and viability dye protocols.

Recently, other viability markers such as platinum compounds and palladium compounds have been assayed to discriminate between live and dead bacteria. These compounds can be chelated in mammalian cells by nucleic acid ligands, are not sensitive to visible light, and are inexpensive compared to monoazide dyes (Karami et al., 2014; Quiroga et al., 1998; Rosenberg et al., 1965, 1969; Soejima and Iwatsuki, 2016). They are able to prevent the detection of purified bacterial DNA and successfully discriminate live and dead *Cronobacter sakazakii* and *Escherichia coli* (Soejima et al., 2016; Soejima and Iwatsuki, 2016), but their suitability to discriminate between infectious and non-infectious viruses has not been evaluated to date.

Other ways of improving viability PCR have been put forward, such as the combination of viability dyes with surfactants (Coudray-Meunier et al., 2013; Moreno et al., 2015) or enzymes like proteinase (Nuanualsuwan and Cliver, 2002; Sánchez et al., 2012), viability marker incubation conditions (Fittipaldi et al., 2012; Nkuipou-Kenfack et al., 2013), or use of a molecular model with a longer amplicon and/or targeting a region rich in RNA secondary structures (Contreras et al., 2011; Coudray-Meunier et al., 2013; Martin et al., 2013; Soejima et al., 2011).

The aim of this work was to develop viability PCR for the discrimination of native and heat-treated NoV and its surrogate MNV. To this end, monoazide dyes, platinum and palladium compounds were screened and viability marker incubation conditions were optimized. Multiple MNV molecular models were designed to evaluate the impact of the targeted region and amplicon length. The optimal viability PCR conditions developed were applied to MNV and to NoV clinical strains to discriminate between native and heat-treated viruses.

2. Materials and methods

2.1. Viruses and cells

Human NoV was obtained from stool samples of infected patients provided and characterized by the National Reference Center (NRC) for Enteric Viruses in Dijon, France (NoV genogroup I stool samples: E1227 GI.2 Southampton; E2818 GI.2; E1488, E8050 and E13194 GI.3; E1509 GI.4 Chiba; E13206 GI.b/I.6. NoV genogroup II stool samples: E7022 GII.3; E1008 GII.4 Lordsdale; E2694 GII.4 Bristol; E6620 GII.4 variant 2010; E6929 and E13085 GII.4; E12990 GII.17; E1077, E1120 and E3135 undetermined). The fecal samples were suspended in phosphate buffered saline, pH 7.4, no calcium, no magnesium (PBS), to obtain a final 10% suspension (w/v), vortexed and centrifuged at 3000*g* for 30 min at 5 °C. In order to decrease the interfering effect of human fecal material, supernatants were filtered through 0.22 µm filters and centrifuged at 5000*g* for 30 min at 5 °C in an Amicon Ultracel-30 K device (Merck Millipore, Nottingham, United Kingdom). Concentrates were collected and reconstituted to the original sample volume in PBS. Aliquots (100 µL) were stored at −80 °C. The titers of the purified fecal suspensions were established in genomic copies with an RT-qPCR standard curve obtained with ten-fold diluted *in vitro* RNA transcripts, as previously described (Fraisse et al., 2017).

MNV-1 (CW1 strain) was provided by Dr. H. Virgin from Washington University (St. Louis, USA) to the ANSES Fougères Laboratory (Fougères, France) and was propagated in a mouse leukemic monocyte macrophage (RAW 264.7, ATCC TIB-71) cell line (Cannon et al., 2006). MNV-1 stock was produced as previously described (Wobus et al., 2004). In order to decrease the interfering effect of the cell culture medium, supernatant was filtered through 0.22 µm filters and centrifuged at 5000*g* for 30 min at 5 °C in an Amicon Ultracel-30 K device (Merck Millipore). Concentrates were collected and reconstituted to the original sample volume in PBS. Aliquots (100 µL) were stored at −80 °C. The titer of viral production was established in genomic copies with an RT-qPCR standard curve obtained with ten-fold diluted *in vitro* RNA transcripts, as previously described (Fraisse et al., 2017).

The NoV GII E6929 strain and MNV-1 RNA were extracted from viral stocks as described in § 2.7. Nucleic acids were eluted and pooled