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Evaluation of methods measuring the capsid integrity and/or functions of noroviruses by heat inactivation

Dan Li[a,*], Leen Baert[a], Ming Xia[c], Weiming Zhong[c], Els Van Coillie[b], Xi Jiang[c], Mieke Uyttendaele[a]

[a] Laboratory of Food Microbiology and Food Preservation, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium
[b] Institute for Agricultural and Fisheries Research, Technology and Food Science Unit, Brusselsesteenweg 370, 9090 Melle, Belgium
[c] Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine, 3333 Burnet Avenue, Cincinnati, OH 45229-3039, USA

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Attempts were made to evaluate methods measuring the capsid integrity and/or functions of noroviruses (NoVs) following heat treatment. Intact viruses (Murine Norovirus-1 [MNV-1] and human NoV GII.4), virus like particles (VLPs) and P particles (expressed in vitro from the protruding domain of the viral capsid) of NoVs were used in this study. Following heat treatment, no significant difference of viral titer of MNV-1 versus NoV GII.4 was observed by RNase One RT-PCR or cell-binding RT-PCR, although cell-binding RT-PCR (to measure the capsid functions) revealed higher reductions than RNase One RT-PCR (to measure the capsid integrity). These results indicate that the function assay for receptor binding is more sensitive than the capsid integrity assay to measure the protected viral RNA. MNV-1 could be used as a surrogate for human NoVs by heat inactivation from the perspective of capsid integrity and/or functions. The heat resistance varied among different GI and GII NoV strains when their P particles were studied.
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1. Introduction

Noroviruses (NoVs), members of the *Caliciviridae* family, are a major cause of acute gastroenteritis worldwide with severe threat to public health and economic burden (Turcios et al., 2006; Koh et al., 2011). Large NoV outbreaks can be caused by viral contaminated food or water, followed by spread of the infection by person-to-person transmission by direct contact or by contaminated environment surfaces (FAO/WHO, 2008). Therefore, the development of procedures for control of the outbreaks by inactivation of NoVs in foods and drinking waters is a priority. Among different food processing techniques, the heat treatment has been most widely used in the food industries area for food safety control and preservation (Rahman, 2007). Due to the customer demands for minimally processed foods to maintain nutrition and flavor, it is of interest to focus on the inactivation of foodborne pathogens with rather short times and low temperatures (Baert et al., 2008a; Uyttendaele et al., 2008).

Because of the lack of suitable animal models and the inability to propagate human NoVs in cell cultures, surrogates that share pathological and/or biological features with human NoVs have been used to study the inactivation of human NoVs (Fino and Kniel, 2008; Hewitt et al., 2009; Li et al., 2011a). Murine Norovirus-1 (MNV-1) has been used as a surrogate as it is cultivable, transmitted enterically, and belongs to the same genus as human NoVs (Wobus et al., 2004).

RT-PCR, which is recognized as the gold standard for human NoV detection, has the drawback of inability to discriminate between infectious and non-infectious viruses (Richards, 1999). Besides the use of surrogates, methods on the evaluation of human NoV infectivity by determining the capsid integrity and functions have been reported (Nuanualsuwan and Cliver, 2002; Parshionikar et al., 2010; Sano et al., 2010). In 2009, Topping et al. (2009) developed a one-step RT-PCR method in combination with RNase treatment in order to examine the effect of temperature on the ability of virus capsids to protect their RNA content. In 2011, a receptor-binding RT-PCR was developed to measuring the ability of host histo-blood group antigen binding of NoVs as indication of infectivity following heat inactivation (Li et al., 2011b). Although neither assay identified a reduction in virus titer comparable to that observed with an infectivity assay, these studies do indicate great improvement in the evaluation of the infectivity of non-cultivable human NoVs compared with the straight RT-PCR.

Virus-like particles (VLPs) of human NoVs, which are expressed in baculovirus-infected insect cells (Jiang et al., 1992), have been shown to have the same morphological, antigenic and glycan binding properties as that of authentic viruses found in human feces (Huang et al., 2005; Murakami et al., 2010). Furthermore, the protruding (P) domain of the major structural protein of NoV capsid VP1 forms subviral particles, the P particles, when the protein is

* Corresponding author. Tel.: +32 9 264 99 29; fax: +32 9 225 55 10.
E-mail address: Dan.Li@UGent.be (D. Li).

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expressed in *Escherichia coli*. The P domain forms the outermost surface of the capsid and contains all elements required for viral capsid binding to host carbohydrate receptors (Tan and Jiang, 2005; Tan et al., 2008). Both VLPs and P particles may be used to study the reaction of human NoVs towards heat treatment, indicating the change of their binding abilities to specific receptors.

In this study, the capsid integrity and/or functions of NoVs by heat inactivation were investigated using natural NoVs from human stools by the RNase pretreated RT-PCR and the receptor-binding RT-PCR. In addition, we performed similar studies on the MNV-1 as a potential surrogate for human NoVs. Finally, also VLPs and P particles from different NoV GI and GII genotypes were studied for their stability to heat treatment detected by receptor-binding ELISA.

2. Materials and methods

2.1. Viruses, viral surrogates and cells

Cells of the murine macrophage cell line RAW 264.7 (ATCC TIB-71; kindly provided by Prof. H.W. Virgin, Washington University School of Medicine, St. Louis, MO, USA) were maintained in complete DMEM medium and grown at 37 °C under a 5% CO_2 atmosphere. Complete DMEM consisted of Dulbecco's modified Eagle's medium (DMEM; Lonza, Walkersville, MD, USA) containing 10% low-endotoxin fetal bovine serum (HyClone, Logan, UT, USA), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin (Lonza), 10 mM HEPES (Lonza), and 2 mM L-glutamine (Lonza).

Cells of the human enterocytic cell line Caco-2 (ECACC 86010202) were cultured in Eagle's minimum essential medium with Earle's salts (EMEM; Lonza) supplemented with 10% low-endotoxin fetal bovine serum (HyClone), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin (Lonza), and 2 mM L-glutamine (Lonza) and grown at 37 °C under a 5% CO_2 atmosphere.

RAW 264.7 cells were infected with MNV-1.CW1, passage 7 at a multiplicity of infection (MOI) of 0.05 (MNV-1: cells) for 2 days. After two freeze–thaw cycles, low speed centrifugation was used to remove cellular debris from the virus suspension, as described by Wobus et al. (2004). The lysate containing suspended MNV-1 was stored in aliquots at −75 °C. The titer of MNV-1 (PFU/ml) was determined by the plaque assay, as described by Wobus et al. (2004).

Fecal suspensions of human NoV GII.4 were kindly provided by the Rega Institute for Medical Research (Leuven, Belgium), diluted with phosphate buffered saline (PBS, Lonza), and stored in aliquots at −75 °C.

Virus-like particles (VLPs) of human NoV GII.4 (VA387), P particles of human NoV GII.4 (VA387), GII.9 (VA207), GI.1 (Norwalk virus), GI.4 (Kobienz 433) were prepared as described previously by Tan and Jiang (2005).

2.2. Viral RNA extraction and RT-PCR

Viral RNA was extracted from 100 μl samples (MNV-1 or human NoVs fecal suspensions) by using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the RNA Cleanup protocol and then stored at −75 °C. The RT-PCR assay was performed as described previously by Baert et al. (2008b) for MNV-1 and Stals et al. (2009) for human NoVs GII.4.

2.3. Heat inactivation

The heat treatments were performed in water bath with set temperatures. The virus or viral surrogate suspensions [cell culture medium for MNV-1, PBS for human NoV GII.4 fecal suspension and 2% of dried milk (Blotto) for human NoVs VLPs and P particles] were subjected to heat treatment in microcentrifuge tubes (500 μl of each). A temperature profile was monitored in parallel in 500 μl of cell culture medium for MNV-1 by a temperature data logger (testo 177-T4, Belgium). All of the heated samples were stored on ice and detected by different methods on the same day.

2.4. Cell-binding RT-PCR

The cell-binding RT-PCR was performed as described by Li et al. (2011b). Briefly, RAW 264.7 or Caco-2 cells were seeded into 24-well plates at a density of 5×10^5 viable cells per well. RAW 264.7 cells were used on the following day, while Caco-2 cells were incubated for at least 7 days post confluency and used as differentiated Caco-2 cells. MNV-1 or human NoVs samples were 10-times diluted in complete DMEM medium (for MNV-1) or PBS (for human NoVs) and then 200 μl per dilution of the MNV-1 or human NoVs sample was plated into 2 wells; 100 μl per well. Inoculated cells were incubated for 1 h at 4 °C and rocked manually every 15 min. The inocula were removed after 1 h incubation and the cells were washed 3 times using PBS. The first washing step was performed by adding 0.5 ml of PBS and by rocking the plates manually. Secondly, the liquid was removed, 0.5 ml of PBS was added again and the cells were scraped off. Suspensions were vortexed and centrifuged at 6000 × *g* for 5 min (Eppendorf 5417C). Supernatant was removed. Thirdly, the pellets were resuspended in PBS, vortexed and centrifuged again. The final pellets were resuspended in PBS (100 μl per sample) and stored at −20 °C until the RNA extraction and RT-PCR was performed.

2.5. RNase One RT-PCR

The RNase One RT-PCR was performed as described by Topping et al. (2009). Briefly, 100 μl of MNV-1 or human NoVs sample was added with 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. Control samples containing RNase buffer but without RNase were kept on ice. All the samples were stored at −20 °C until the RNA extraction and RT-PCR was performed.

2.6. Combination of RNase One and cell-binding RT-PCR

MNV-1 or human NoVs sample was plated onto RAW 264.7 or differentiated Caco-2 cells, incubated, washed and collected as described in the part cell-binding RT-PCR. The final pellets were resuspended in PBS (100 μl per sample) and treated by RNase One as described in the part of RNase One RT-PCR. All the samples were stored at −20 °C until the RNA extraction and RT-PCR was performed.

2.7. Saliva-binding enzyme-linked immunosorbent assay (ELISA)

The saliva-binding ELISA was performed as described by Huang et al. (2005). Briefly, saliva samples of known types (Table 1) were boiled at 100 °C and centrifugation at 10,000 × *g* for 5 min. The supernatant was then used to coat 96-well microtiter plates (Dynex Immulon; Dynatech, Franklin, MA, USA) at a dilution of 1:500 in PBS at 4 °C overnight. The non-coated wells were included as negative controls. After blocking with 5% non-fat dried milk (Blotto), treated samples with a certain concentration (Table 1) were added. The bound capsid proteins were detected with hyperimmune guinea pig anti-NoV antisera (1:3300), and by adding horseradish peroxidase-conjugated goat anti-guinea pig immunoglobulin G (1:2000, ICN, Aurora, OH, USA). The horseradish peroxidase activity was detected with a TMB kit (Kirkegaard & Perry Laboratories,

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