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Evaluation of heat treatment for inactivation of norovirus genogroup II in foods

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Abstract

The effective food processing technology is a key step in eliminating human noroviruses in foods mainly due to their stability in diverse environmental conditions. The aim of this study was to evaluate the effect of rising temperatures for inactivation of norovirus genogroup (G) II and murine norovirus 1 in samples of tomato sauce (72–74 °C for 1 min) and ground meat (100 °C for 30 min). Spiking experiments were carried out in triplicate using TRIzol® reagent method associated with quantitative polymerase chain reaction (qPCR) TaqMan™ system combined with previous free RNA digestion. Success rate and efficiency recoveries of both viruses as well limit of detection of a method for each matrix were also conducted. The heat treatment applied here proved to be efficient to reduce the burden of norovirus GII in a range of 1–4 $\log_{10}$ genomic copies per gram (percentage ranging from 0.45 to 104.54%) in both matrices. The experiments in this study showed that the results of norovirus GII and murine norovirus 1 in tomato sauce and ground meat tested during thermal treatments cannot be generalized to other food matrices, since there may be food-specific protective effects, as the presence of different components, that can interfere in virus inactivation. Studies using different food matrices reinforce the importance to investigate viruses' inactivation thermal processes in foods due to the resistance of these viruses to adverse conditions, contributing to food security in food virology.

Keywords Human norovirus GII · Murine norovirus 1 · Thermal inactivation · Tomato sauce · Ground meat · Food virology

Introduction

Viruses have been described as a leading cause of foodborne diseases [1] and, although a wide variety of food-related viruses exists, noroviruses are the most important since it is related to a large number of gastroenteritis outbreaks worldwide [2, 3]. Resistance to adverse environmental conditions and the low infectious dose of those viruses means that even a small amount of contamination is a potential threat to public health [4].

Norovirus belongs to the family *Caliciviridae*, genus *Norovirus*, formerly Norwalk-like virus, divided into ten genogroups (GI to GX) which GI, GII, GIV, GVIII, and GIX can origin disease in humans [5]. They are non-enveloped icosahedral viruses with a single-stranded positive-sense linear RNA genome, organized into three open reading frames (ORFs) [6–9].

Norovirus can be transmitted through several means, including consumption of contaminated food, person-to-person contact, exposure to aerosolized vomitus from an infected person, or by touching contaminated surfaces or fomites [10, 11]. Infections occur all-year-round, causing gastroenteritis in people of all ages. The greatest public health impact from norovirus outbreaks has been reported in institutions such as hospitals and nursing homes, where norovirus outbreaks commonly occur due to the close proximity of patients in an enclosed environment [8].

Since enteric viruses as norovirus must survive the enzymatic and extreme pH conditions in the gastrointestinal tract to infect a host, they tend to be resistant to a wide range of commonly used food processing treatments and can persist in foods for days and weeks in most environments without loss of infectivity [12].

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Heat treatment is a traditional and an industrial way of processing and keeping food safe, and most studies on physicochemical stability profiles include the evaluation of thermal inactivation [13, 14]. Viruses are exposed to temperatures and time intervals used in the food industries, such as pasteurization, or even in retail circumstances (e.g., food service establishments, restaurants) and, subsequently, evaluated for stability under these conditions [15].

In those studies, once there are no routine cell culture systems available for human norovirus, different viruses have been evaluated as surrogates, including murine norovirus 1 [4, 16–21]. When evaluating risk management options, the use of a surrogate will not always mimic the resistance of the intended foodborne viruses [8].

The effects of heat treatment on virus infectivity in foods are highly dependent on the virus (sub)-type, food matrix, and the initial level of viral contaminants. However, light cooking, e.g., steaming, searing, may not be adequate to inactivate viral infectivity, thus leading to unsafe foods. Moreover, given the potential for contamination with millions of viral particles and an infectious dose as low as a few viral particles, even conventional pasteurization may not adequately inactivate norovirus in contaminated foods [22].

Molecular methods such as quantitative reverse transcription–real-time polymerase chain reaction (qPCR) are currently the most widely used technologies for the detection of norovirus in food samples [23–25]; however, they cannot be relied upon to distinguish between infectious and inactivated viruses [26–28]. To discriminate the integrity of viral particles, RNAse or propidium monoazide treatments preceding qPCR have been successfully used [29–31].

Inactivation and infectivity of norovirus are challenging issues, so we do not intend to exhaust the topic but present results obtained by conducting experimental studies of artificial contamination of human norovirus GII "in natura" and industrialized foods.

The aim of this study was to evaluate the inactivation of human norovirus GII by heat treatment in tomato sauce and ground meat assessing thermal and time conditions similar to those used in industrial food processing methods such as pasteurization and in food preparation (cooking) by consumers.

Materials and methods

Food samples and virus strains

Tomato sauce and ground meat samples were obtained from local food stores in the state of Rio de Janeiro, Brazil. Human norovirus GII.4 strain was obtained from 10 to 20% fecal suspension in Tris/HCl/Ca^{+2} buffer (pH 7.2–7.3), previously screened and characterized by nucleotide sequencing at the Laboratory of Comparative and Environmental Virology collection at the Oswaldo Cruz Institute, Rio de Janeiro-RJ, Brazil. Murine norovirus 1 was kindly provided by Dr. Herbert W. Virgin from Washington University School of Medicine and propagated in RAW 264.7 cells (a macrophage-like Abelson leukemia virus-transformed cell line derived from BALB/c mice) according to de Abreu Corrêa and Miagostovich (2013) [32].

Elution-concentration method

TRIzol® reagent in a 400 mL polypropylene bag with filter compartment (Nasco®, Fort Atkinson, Wisconsin, USA) was used for recovering viruses from matrices as previously described by Schwab et al. (2000) [33], with modifications. Twenty-five grams of tomato sauce and ground meat samples separately were homogenized with 20 mL of TRIzol® reagent (Sigma-Aldrich, St. Louis, USA), allowing a contact time on shaking for 20 min and further centrifugation performed for 30 min at 4000 × *g*, 4 °C. The final recovered samples (supernatant) were taken and stored at − 20 °C until viral RNA extraction from 140 µL of the concentrated samples.

Limit of detection

Independent spiking experiments were carried out in triplicate for norovirus GII and murine norovirus 1 to determine the detection limit by serial dilutions of the viruses (10^{0}–10^{-8} genomic copies/gc) in phosphate buffer (PBS, Na_2HPO_4 0.2 M/NaH_2PO_4 0.2 M; 1:2 *v*/*v*; pH 7.5), and the optimal amount. After inoculation of norovirus GII and murine norovirus 1 at the same time in 25 g of each (tomato sauce and ground meat samples) for nearly 2 h at room temperature, they were concentrated with TRIzol® reagent as previously described ("Elution-concentration method" section).

Viral RNA extraction, reverse transcription, and virus detection by qPCR

RNA was extracted using the QIAamp Viral RNA Mini Kit® (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The reverse transcription reaction was performed at 50 °C for 1 h, and the enzyme was then inactivated at 70 °C for 20 min. For murine norovirus 1 and human norovirus GII quantification, qPCR was performed using a TaqMan™ system (Applied Biosystems, Foster City, CA, USA) and a set of primers and probes described by Baert et al. (2008)b [34] and Kageyama et al. (2003) [35], respectively. Both undiluted and 1:10 diluted RNA samples were analyzed in duplicate, totaling four qPCR reactions per sample. These samples were considered positive when at least one replica was detected at the threshold cycle (Ct) of ≤ 38.

Spiking experiments to assess thermal inactivation of human norovirus GII and murine norovirus 1

After analysis of detection limit, 25 g of tomato sauce and ground meat were contaminated experimentally with 100 µL of murine norovirus 1 lysate, corresponding with 10^6 gc, in a 400-mL polypropylene bag with filter compartment (Nasco®, Fort Atkinson, Wisconsin, USA) separately to each matrix and only stored at 4 °C for 24 h as a sample process control virus. During incubation of food products inoculated with murine norovirus 1 at 4 °C, the samples were mixed five times manually in a period of approximately 20 min. These procedures were adapted according to Mormann et al. (2010) [36].

After this period, 250 µL of the tenfold dilution of a fecal sample of norovirus GII (corresponding with around 10^4 gc) was added to the tomato sauce and processed to temperatures ranging from 72–74 °C for 1 min at water bath (FANEN 515, São Paulo, Brazil), simulating pasteurization. For ground meat, the samples were processed at a heating temperature of 100 °C in a drying oven (FANEN 515, São Paulo, Brazil) for approximately 30 min. Non-contaminated food samples, only diluted with PBS (pH 7.5), were also processed as negative controls in all experiments. The experiments were performed in triplicate and independently.

After heat treatment, food samples were processed using the TRIzol® reagent method. Before viral RNA extraction, 140 µL of concentrated were treated by adding 35 µg of RNase A (Qiagen, Valencia, CA, USA) per sample and incubated at 37 °C for 1 h. To avoid residual RNAse activity, 50 U per RNAse inhibitor sample (Invitrogen®, USA) was also added, and the mixture was incubated for 30 min at room temperature. Negative RNAses treatment control samples were processed for each matrix.

Data analysis

Success rate (qualitative) and efficiency recovery (quantitative) analyses were performed according to Stals et al. (2011) [37]. Recovery success rates were calculated as "the number of PCR reactions determining successful recovery of viruses" per "number of PCR reactions performed," while recovery efficiencies considered "the mean recovered a number of gc" per "mean inoculated number of gc." The results of the recovery rates were expressed as an average of the assays.

Results and discussion

Tenfold serial dilutions of murine norovirus 1 and human norovirus GII were performed in PBS (pH 7.5) to evaluate viral detection limit, being 3.08×10^2 and 1.06×10^1 gc/g, respectively.

Table 1 presents the results regarding the success rate and the efficiency of viral recovery according to the matrices used. As noted, the viral recovery success rate was 100% for murine norovirus 1, regardless of the food matrix analyzed. For norovirus GII, a lower rate was observed when using tomato sauce as a matrix (70.83%). Murine norovirus 1 was the first norovirus to be propagated in cell culture once shares similar genetic and structural features with human noroviruses [20], being used as a human norovirus surrogate [38–40].

A higher recovery efficiency (GII: 20.78%; murine norovirus 1: 24.04%) from tomato sauce when compared to recovery from ground meat (GII: 8.53%; murine norovirus 1: 4.63%) was observed, regardless of the virus analyzed (Table 1). When the different food matrices were heated according to the different temperatures proposed, a decrease in the success rate of recovery regardless of the matrix was noted, but especially concerning norovirus GII. However, in relation to recovery efficiency, different results were observed in the different food matrices. In relation to tomato sauce, heating resulted in a decrease in the average recovery efficiency (GII: 14.15%; murine norovirus 1: 11.41%), while for ground meat, there was a considerable increase in viral recovery efficiency, regardless of virus (GII: 62.29%; murine norovirus 1: 19.54%).

Table 1 Success rate and efficiency recoveries of norovirus GII and murine norovirus 1 using TRIzol® method according to food matrix and heat treatment

Food matrix	Virus	No heat		Heat	
		Success rate Positive (%)	% Efficiency (mean range)	Success rate Positive (%)	% Efficiency (mean range)
Tomato sauce[a]	GII	17 (70.83)	20.78 (17.30–27.25)	9 (37.5)	14.15 (6.43–21.87)
	MNV-1	24 (100.00)	24.04 (14.21–42.05)	24 (100.00)	11.41 (0.01–28.47)
Ground meat[b]	GII	23 (95.83)	8.53 (3.67–26.22)	15 (62.5)	62.29 (22.21–115.21)
	MNV-1	24 (100.00)	4.63 (2.19–11.31)	20 (83.33)	19.54 (4.97–49.55)

Heat treatment: [a]72–74 °C/1′; [b]100 °C/30′

Heat treatment using experimental conditions similar to pasteurization and cooking revealed human norovirus GII and murine norovirus 1 reduction with variations in different matrices ranging from 1 to 4 $\log_{10}$, corresponding to a different percentage of reduction (Table 2). Different methods for in vitro thermal inactivation include features such as the choice of temperature and time point, the type of food, medium or buffer (matrices), the type of heat (dry vs. wet based), the type of infectivity analysis as well as the volume and concentration of virus analyzed [41]. Therefore, these features were carefully considered here to assess human norovirus GII as well as murine norovirus 1.

Methods to extract viral nucleic acids as TRIzol® reagent, used here as a viral recovery method, has been described to render a non-infectious virus detection [42–44], once this reagent contains a chaotropic salt (guanidine isothiocyanate), which acts to denature macromolecules.

Despite the latest description that genetically encoded host factors are necessary for infection and cell culture of noroviruses [45], culture-independent methods, more specifically RT-qPCR, are still used for virus detection and quantification. However, an important limitation of this method is its inability to differentiate 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, frequently underestimating the efficacy of a given inactivation strategy [46, 47].

Our results revealed not only variation regarding virus as well as food matrices when the same heat treatment was applied since individual food-specific protective effects may influence viruses' inactivation, making a generalization of results unfeasible. This reinforces the importance of further studies on norovirus inactivation in varied food matrices and robust statistical analyzes. Factors such as inoculum level added to the food, composition of the food matrix, the role of the temperature, viral recovery method may contribute to variations on data obtained [37, 41].

The role of temperature in the recovery efficiency of viruses was evident (Table 1). When the data obtained in the tomato sauce was analyzed, we observed that pasteurization temperature reduced the recovery efficiency of both viruses. However, the same was not observed for ground meat when the cooking temperature was simulated. Comparisons cannot be made here due to the difference in matrices and heating treatment; however, we can question what resulted in an increase in virus recovery after cooking the meat for 30 min.

Factor as homogeneous exposure to heating temperature and complexity of the ground meat matrix inoculated experimentally with viruses could explain our results. According to [4], heat inactivation of viruses could change the capsid of the virus particle, despite the matrix may protect the viruses from the heat treatment [36]. It is important to highlight in our study that murine norovirus 1 was subjected to heating in its original cell lysate, while human norovirus GII was subjected to PBS-diluted fecal suspension, which may have difficult components [48].

Analyses in duplicate for qPCR to 1:10 diluted RNA did not represent an improvement in inhibition of compounds, probably due to the complexity of food matrices (tomato sauce and ground meat) and possible protective effects on the viral particles in these products.

This study also demonstrated that the use of the RNAse enzyme revealed the evidence of infectious viruses after processing even in a low percentage, thus corroborating previous studies that demonstrated that inactivation of the infectious virus may be incomplete during viral nucleic acid extraction by TRIzol® reagent [43].

The abilities of different enzymatic pretreatments for removing damaged, non-infectious viral particles from samples containing viruses prior to RT-PCR have been widely tested. Another approach is to pre-treat with nucleic acid intercalating agents like propidium monoazide (PMA) or ethidium monoazide (EMA) [31, 49–53].

Fraisse et al. (2018) [54] developed a viability PCR method to discriminate between native and heat-treated virus for norovirus and its surrogate, murine norovirus, and screening of viability markers (monoazide dyes, platinum, and palladium compounds) was performed on viral RNA. The authors found the viability PCR discriminated efficiently between native and heat-inactivated murine norovirus at 72 and 80 °C, and efficiently reduced the genomic titer of heat-treated norovirus strains.

Capsid damage appears to be the most common reason for rendering noninfectious viruses [55] once capsid protects the viral genome from nucleases action [56, 57]. The discrepancy was observed in the samples treated with

Table 2 Percentage and logarithmic reduction (mean range) of human norovirus GII and murine norovirus 1 after heat treatment according quantification preceded without (W/o) or with (W/) RNAse treatment

Food matrices	% (Log reduction – mean range)			
	Human norovirus GII		Murine norovirus 1	
	W/o RNAse	W/ RNAse	W/o RNAse	W/ RNAse
Tomato sauce (72–74 °C/1′)	51.72 (2–4)	0.45 (2–4)	26.89 (2–4)	1.07 (2–4)
Ground meat (100 °C/30′)	104.54 (1–4)	2.36 (2)	214.73 (3–4)	1137.69 (3–4)

RNAses, striving evaluation of viral inactivation, possibly by the processing that the ground meat was submitted previously, human norovirus GII inoculum on this matrix, heating temperature, reagent TRIzol®, beyond pre-treatment of viral RNA.

Concluding, the heat treatment usually employed for processing and keeping food safe proved to be efficient to reduce the burden of human norovirus. Koopmans and Duizer (2004) [58] classified the risk of infection for the consumer as negligible and low if the reduction in the virus titer is at least 4 $\log_{10}$ and 3 $\log_{10}$, respectively. However, the differences in food matrices studied reinforce the importance to investigate viruses' inactivation processes in foods, contributing to further analysis concerning risk assessment of food contamination and food security.

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Author contribution Formal analysis and investigation, ISL; writing—original draft preparation, ISL; writing—review and editing, MPM; funding acquisition, MPM; supervision, MPM.

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Declarations

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Conflict of interest The authors declare no competing interests.

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