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ARTIGO

Recovery of Norovirus from lettuce (*Lactuca sativa*) using an adsorption — elution method with a negatively charged membrane: comparison of two elution buffers

Recuperação de Norovirus a partir de alface (*Lactuca sativa*) utilizando um método de adsorção-eluição com membrana negativamente carregada — comparação de dois tampões de eluição

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ABSTRACT

Phosphate saline buffer (PBS) and glycine buffer (GB) were evaluated as elution buffers in an adsorption-elution method using a negatively charged membrane associated with quantitative polymerase chain reaction (qPCR) and semi-nested PCR for detection of Norovirus genogroup II (NoV GII) from lettuce. In this methodology, PP7 bacteriophage was used as a virus sample for process control. The qPCR showed more sensitivity than semi-nested PCR for NoV GII detection. The recovery efficiency, using PBS and GB, ranged from 24.72 to 60.78% and 19.48 to 137.26% for NoV GII, and from 0.01 to 0.15% and 0.13 to 6.04% for PP7 bacteriophage, respectively. Elution with GB was more efficient for PP7 bacteriophage recovery ($p = 0.03$), but no difference was seen for NoV GII ($p = 0.57$). The GB performed better than PBS as an eluent solution and can be considered a methodological improvement.

KEYWORDS: Norovirus; Filtration; Lettuce; PP7 Bacteriophage; Internal Control Process

RESUMO

Salina tamponada fosfatada (STF) e tampão glicina (TG) foram avaliados como eluentes em um método de adsorção-eluição, utilizando membrana carregada negativamente associada com reação quantitativa de polimerase em cadeia (qPCR) e semi-nested PCR, para detecção de Norovírus do genogrupo II (NoV GII) a partir de alface. Nesta metodologia, o bacteriófago PP7 foi utilizado como controle do processo. A qPCR apresentou maior sensibilidade do que o semi-nested PCR na detecção de NoV GII. A eficiência de recuperação, utilizando STF e TG, variou de 24,72 a 60,78% e de 19,48 a 137,26% para NoV GII e de 0,01 a 0,15% e de 0,13 a 6,04% para o bacteriófago PP7, respectivamente. A eluição com TG foi mais eficiente na recuperação do bacteriófago PP7 ($p = 0,03$) embora nenhuma diferença tenha sido observada para NoV GII ($p = 0,57$). O TG apresentou melhor desempenho que a STF como solução para eluição e pode ser considerada uma melhoria do método.

PALAVRAS-CHAVE: Norovírus; Filtração; Alface; Bacteriófago PP7; Controle Interno de Processo

Introduction

Consuming leafy green vegetables provides important vitamins, minerals, and phyto-nutrients, which are considered important components of a healthy diet[1]. Because of these benefits, governments around the world have encouraged consumption of vegetables to prevent diseases[2]. However, there has been an increased recognition of foodborne disease outbreaks linked to ready-to-eat (RTE) vegetables[3]. In 2008, an expert meeting was organized by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) to consider how adequately to address the scientific advice on microbiological hazards associated with fresh produce. This meeting identified leafy green vegetables as the commodity group of highest concern from a microbiological safety perspective, and Norovirus (NoV) was included among the more common pathogenic microorganisms that can be transmitted to humans through food consumption[4].

Norovirus is a major cause of acute gastroenteritis worldwide and is responsible for up to 1.1 million hospitalizations with an estimated mortality of approximately 218,000 deaths annually[5]. The Norovirus genus belongs to the *Caliciviridae* family and is divided into five genogroups (G), of which GI, II, and IV are known to infect humans[6]; GII is the most prevalent among cases of foodborne infections[7].

The impact of foodborne viral diseases is increasingly recognized, and the FAO/WHO has signaled an upward trend in their incidence. This is particularly pertinent to vegetables that in general are not cooked before consumption. Lettuce has been acknowledged specifically as a source of NoV infection because it is a RTE food that can be consumed raw in salads[3].

To improve microbiological monitoring of food quality and assess food's true role in viral transmission, new approaches have focused on virus extraction, concentration, and detection using molecular technology to improve methodological sensitivity[8,9]. Viral elution with neutral or alkaline buffers before a concentration step using polyethylene glycol (PEG) precipitation, ultracentrifugation, or negatively charged filters has already been reported[10-13]. These methods are generally associated with amplification of viral RNA by reverse transcription and quantitative polymerase chain reaction (qPCR) and thus is currently considered the most sensitive, widely used method for detecting NoV in food samples[13]. However, this technology is not yet accessible to all official food control laboratories, especially in developing countries[14].

This study evaluated the use of phosphate buffer saline (PBS) and glicine buffer (GB) as elution solutions in an adsorption-elution concentration method for recovering NoV GII from lettuce (*Lactuca sativa*), using negatively charged membranes associated to a qPCR and semi-nested PCR[11,15,16]. PP7 bacteriophage was used as a virus sample for process control (SPCV — sample process control virus), since it is regarded as a suitable surrogate for human enteric viruses from water samples[17,18].

Methodology

Viruses

Norovirus (Hawaii virus) GII.1 strain prototype and PP7 bacteriophage (ATCC 15692-B2) were used for constructing the quantitative assays' standard curve (SC). For spiking experiments, titers of NoV GII (10% (w/v) positive fecal suspension) and PP7 bacteriophage particle suspensions were established by real time PCR based on SC, represented by the absolute number of genome copies (GC) μl^{-1}.

Evaluation of NoV GII and PP7 Bacteriophage Recovery from Lettuce Artificially Contaminated Using Different Eluting Buffers

An experiment using different inoculum concentrations of viruses was conducted to evaluate the recovery efficiency of PP7 bacteriophage and NoV GII through qPCR using PBS (pH 7.2) and GB (0.3 M NaCl, 0.1 M glycine, pH 9.5) as elution solutions. In addition, the recovery success rate of NoV GII by semi-nested PCR was also verified.

Briefly, aliquots of 25 g of the same minimally processed lettuce sample were seeded by direct application of 50 µl of NoV GII fecal suspension or its serial dilutions (10^{-1}, 10^{-2}, 10^{-3}), while others were seeded by direct application of the PP7 bacteriophage particle suspension or its serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) onto the food surface. Those RNA virus samples remained for 30 min in a laminar flow hood to facilitate viruses' attachment. Viruses were concentrated by an adsorption-elution method using negatively charged membranes, as previously described by Fumian et al.[11] with one modification. The centrifugation step was subtracted, and the samples were weighed in sterile Whirl-Pak® Stomacher filter bags (Nasco®, Fort Atkinson, Wisconsin, USA) and homogenized in a Stomacher® apparatus, as described by other authors[9,12,13,19]. The rinse fluid in the filter compartment of the bag was used to perform the analysis. Part of the NoV GII fecal suspension, the PP7 bacteriophage suspension, and its respective dilutions were applied to RNA extraction and quantification, together with the contaminated aliquots. The PP7 bacteriophage was used as SPCV because of its similarity in size (25 nm) and in physicochemical properties to poliovirus, simulating the worst scenario for viral filtration[18]. Unspiked samples served as negative controls. All viral experimental assays (including negative controls) were separately conducted in triplicate for each type of virus and elution solution.

RNA Extraction and cDNA Synthesis

Viral RNA was extracted from 140 µl of the 2 mL final eluate using a QIAamp® viral RNA mini kit (Qiagen®, Valencia, CA, USA), in accordance with the manufacturer's instructions, to obtain a final volume of 60 µl. The High-Capacity kit (Applied Biosystems,

Foster City, CA, USA) was used for cDNA synthesis. In each reaction, 12.5 µl of viral RNA extract were added to 12.5 µl of RT reaction mixture containing: 1x buffer, 8 mmol l^{-1} of each dNTP, 62.5 U of MultiScribe™ reverse transcriptase, and 2x random primers. The reverse transcription conditions were performed as follows: 10 min at 25ºC, 2 h at 37ºC, and 5 min at 85ºC. For each reaction setup, negative (DNA/RNA free water — BioBasic, Ontario, Canada) and positive (NoV GII or PP7 bacteriophage) controls were included. To investigate the presence of inhibitors in samples, cDNA was also prepared using a 1:10 RNA dilution.

Norovirus GII and PP7 Bacteriophage Detection/ Quantification

Norovirus GII and PP7 bacteriophage detection was conducted using a TaqMan® technology of qPCR, according to protocols previously described[15,17,18]. Primers and probes are shown in Table 1. Reactions were performed in duplicate, using the ABI 7500 Real-Time PCR System (Applied Biosystems®, Foster City, CA, USA) according to the manufacturer's instructions. The generation of plasmids and the construction of the SC were performed as previously described[11,20]. The SC was created using tenfold serial dilutions of PCR®2.1-TOPO vectors (Invitrogen®, Carlsbad, CA, USA), containing either the ORF1/ORF2 overlap region of the NoV genome (5.0×10^6 to 5.0×10^0) or the PP7 replicase gene (1.0×10^7 to 1.0×10^1).

Semi-nested PCR was also performed to detect NoV GII, using primers JV13I, JV12Y, and NoroII-R, inner primer specific for GII genotypes (Table 1) that target the viral RNA-dependent RNA polymerase gene[16]. All procedures comprised negative (DNA/RNA free water — BioBasic, Ontario, Canada) and positive (NoV GII and PP7 bacteriophage) controls to avoid false results; four separate rooms were used to perform pre amplification and post amplification reactions and manipulations.

Data Analysis

Recovery of NoV GII and PP7 bacteriophage were both quantitatively and qualitatively analyzed as described by Stals et al.[13]. Quantitative analysis ("recovery efficiency") was calculated by comparing the mean of NoV GII or PP7 bacteriophage GC recovered with the mean of GC inoculated. Qualitative analysis ("recovery success rate") of NoV GII or PP7 bacteriophage recovery was performed by comparing the number of positive qPCR signals with the total number of reactions. A qualitative analysis of semi-nested PCR for NoV GII detection was also conducted. The Mann-Whitney test (MW-test) was used to evaluate the effect of virus elution with PBS and GB, comparing the median values of PP7 bacteriophage and NoV GII recovery efficiency.

Results

Table 2 shows the performance of PBS and GB for PP7 bacteriophage and NoV GII recovery. Quantitative analysis showed that PP7 bacteriophage recovery efficiency ranged from 0.01 to 0.15% and from 0.13 to 6.04% using PBS and GB, respectively (Table 2). The minimum and maximum recovery efficiency was observed at a dilution of 10^{-1} and 10^{-3}, respectively, using both solutions. The recovery efficiency of NoV GII ranged from 24.72 to 60.78% and from 19.48 to 137.26% using PBS and GB, respectively (Table 2). The GB showed better recovery efficiency of PP7 bacteriophage than did PBS (MW-test; $p = 0.03$). However, no significant difference was observed for NoV GII (MW-test; $p = 0.57$) recovery. Negative controls did not show any amplification.

Semi-nested PCR qualitative analysis showed that the high NoV GII inoculum level could be recovered from all samples seeded using both buffers (Table 3). Nevertheless, the use of higher dilutions decreased success rates, especially when PBS was used. Although NoV GII inoculum was not detected using PBS, a low level could be recovered using GB, with a success rate of 2/3 and 1/3 using RNA and a 1:10 RNA dilution, respectively (Table 3). The use of a 1:10 RNA dilution presented reduction of recovery success rates for both elution buffers (Table 2). The minimally processed lettuce sample used during this experiment did not show intrinsic contamination for NoV GII.

Table 1. Primers and probes used for qPCRs and nested PCR performed in this study.

Molecular method	Virus	Primer/Probe	Sequence (5'→3')	Location
Real-time quantitative PCR	NoV GII	COG2F	CARGARBCNATGTTYAGRTGGATGAG	5003[a]
		COG2R	TCGACGCCATCTTCATTCACA	5100
		RING2-TP	FAM-TGGGAGGGCGATCGCAATCT-TAMRA	5048
	PP7	247 f	GTTATGAACCAATGTGGCCGTTAT	247[b]
		320 r	CGGGATGCCTCTGAAAAAAG	320
		274 probe	FAM-TCGGTGGTCAACGAGGAACTGGAAC-TAMRA	274
Nested PCR	NoV GII	JV13I	TCATCATCACCATAGAAIGAG	4585[c]
		JV12Y	ATACCACTATGATGCAGAYTA	4279
		NoroII-R	AGCCAGTGGGCGATGGAATTC	4495

Degenerate primers and probes are as follows: Y, C or T; R, A or G; B, not A; N, any. W, A or T; K, G or T; S, G or C.
[a] Corresponding nucleotide position in PP7 bacteriophage (accession number NC_001628).
[b] Corresponding nucleotide position in human NoV (accession number AF145896).
[c] Corresponding nucleotide position in human NoV (accession number M87661).

Table 2. Comparison of elution efficiency between PBS and GB for virus recovery from lettuce.

Inoculum	Eluent	Dilutions	No. of inoculated copies (×10⁴)	Recovery copies in concentrate (mean ± SD[a]) (×10⁴)	Recovery efficiency[b] (mean ± SD) (Recovery success rate)[c]
Bacteriophage PP7	PBS[d]	10^{0}	10,82,142.86	196.27 ± 35.48	0.02 ± 0.003% (3/3)
		10^{-1}	92,250.00	9.15 ± 2.27	0.01 ± 0.002% (3/3)
		10^{-2}	6,578.57	4.00 ± 4.73	0.06 ± 0.07% (3/3)
		10^{-3}	155.36	0.24 ± 0.30	0.15 ± 0.19% (3/3)
	GB[e]	10^{0}	1,082,142.86	30,461.57 ± 52,631.53	2.81 ± 4.86% (3/3)
		10^{-1}	92,250.00	123.51 ± 109.50	0.13 ± 0.12% (3/3)
		10^{-2}	6,578.57	15.03 ± 7.84	0.23 ± 0.12% (3/3)
		10^{-3}	155.36	9.39 ± 6.94	6.04 ± 4.47% (3/3)
NoV GII	PBS	10^{0}	1,264.31	730.64 ± 676.40	57.79 ± 53.50% (3/3)
		10^{-1}	144.02	51.28 ± 21.06	35.61 ± 14.62% (3/3)
		10^{-2}	9.10	2.25 ± 2.02	24.72 ± 22.16% (3/3)
		10^{-3}	0.94	0.57 ± 0.62	60.78 ± 66.21% (2/3)
	GB	10^{0}	1,264.31	900.78 ± 340.13	71.25 ± 26.90% (3/3)
		10^{-1}	144.02	28.06 ± 31.91	19.48 ± 22.16% (3/3)
		10^{-2}	9.10	8.44 ± 9.99	137.26 ± 110.60% (3/3)
		10^{-3}	0.94	0.26 ± 0.28	27.90 ± 29.67% (2/3)

[a] standard deviation; [b] (# genomic copies recovered from concentrate x 100)/# genomic copies inoculated on 25 g of lettuce sample; [c] # Positive real-time PCR reactions/# performed real-time PCR reactions; [d] phosphate buffer saline; [e] glycine buffer.

Discussion

Evaluation of the virus concentrated method using different virus inoculum concentrations, and testing PBS and GB as elution solutions, the NoV GII recovery efficiency using PBS was higher than Fumian et al.[11] reported. Using an inoculum level of 1264.31 x 10^4 GC, the recovery efficiency observed was 57.79% (Table 2) while the recovery observed by the authors using a similar inoculum (1913.24 x 10^4 cg) was 5.2%. The best performance may be associated with the use of filter bags instead of the centrifugation step. This probably happened because some virus particles can be lost throughout centrifugation. Hence, the use of filter bags might be a good alternative for reducing this loss. The NoV GII recovery efficiency observed in this study resembled those reported by authors using other methods for recovering NoV in lettuce, presenting high percentages of variability[8,9,12,13,21].

The low levels of NoV particles and the presence of inhibitors in food matrices usually make its detection difficult and reinforce the need to use SPCV from viral concentration procedures in molecular methodologies of detection and quantification. In this study, PP7 bacteriophage was analyzed to evaluate its reliability as SPCV of the concentration method, and at the end of the viral concentration procedure, it was detected efficiently, showing a recovery success rate of 100% (Table 2). This study focused on PP7 bacteriophage propagation over other similar NoV viruses, such as Murine Norovirus 1 (MNV-1) or feline calicivirus (FCV), since *Pseudomonas aeruginosa* culture required for PP7 bacteriophage propagation is more accessible to food microbiology laboratories than cell cultures used to produce MNV-1 and FCV stocks[17,18,22]. Mengovirus, another virus used as SPCV in studies with oysters and blue mussels as matrices, has also been evaluated, but still requires a laboratory structure used for production of cell culture[23]. The recovery

Table 3. Comparison of elution efficiency between PBS and GB for NoV GII detection using RNA and a 1:10 dilution of RNA by semi-nested PCR.

Eluent	Dilutions	No. of inoculated copies (×10⁴)	Recovery success rate[a]	
			RNA 10^{0}	RNA 10^{-1}
PBS[b]	10^{0}	1,264.31	3/3	3/3
	10^{-1}	144.02	3/3	3/3
	10^{-2}	9.10	2/3	0/3
	10^{-3}	0.94	0/3	0/3
GB[c]	10^{0}	1,264.31	3/3	3/3
	10^{-1}	144.02	3/3	2/3
	10^{-2}	9.10	2/3	2/3
	10^{-3}	0.94	2/3	1/3

[a] # Positive semi-nested PCR reactions/# performed semi-nested PCR reactions; [b] phosphate buffer saline; [c] glycine buffer.

efficiency of PP7 bacteriophage was lower than NoV GII in all concentrations tested (Table 2). One hypothesis is the presence of free RNA and defective viral particles in PP7 bacteriophage suspension used to spike experiments; these can be detected when subjected to nucleic acid extraction, but are more susceptible to elimination during the concentration step, leading to an underestimated calculation of the PP7 bacteriophage recovery efficiency. Furthermore, PP7 bacteriophage adsorption to solids can occur, lowering overall recovery[17] and clogging filters with a large quantity of debris that may be present in the lettuce sample, thus reducing the recovery rate[24].

Glycine buffer performed better than PBS in recovery efficiency of PP7 bacteriophage and of NoV GII using semi-nested PCR, especially when the viral concentration in the lettuce samples was low. These results resembled those of Corrêa and Miagostovich[21] who obtained better results using GB in the recovery of MNV-1, as compared to PBS. Acidic vegetables, such as lettuce, can reduce the extraction solution's pH during the elution step, and this may cause pH to drop below neutrality (10). This pH reduction can induce acid precipitation of viral particles and lead to loss during the processing step[12]. Thus, the use of washing solutions with a buffering capacity is crucial for acidic food products such as lettuce. GB presents a pH buffering area superior to that of PBS, helping to maintain pH of lettuce after homogenization, preventing acid precipitation of viral particles[13,21]. The acidic environment can impair virus elution, and in food products that contain acidic substances, such as vegetables, an alkaline buffer system is recommended[12,19]. Furthermore, the higher pH range (9.5) and the greater ionic strength, with twice more NaCl than PBS, can break the electrostatic and hydrophobic interactions between vegetable surfaces and viruses[10]. Sánchez et al.[9] did not observe differences between Tris-glycine-beef extract buffer (100 mM Tris, 50 mM glycine, 1% (wt/vol) beef extract, pH 9.5) and buffered peptone water (pH 7.2), suggesting that the presence of NaCl is important for extracting viral particles. However, Tian et al.[25] reported that water (pH 8.0) and PBS (pH 7.4) showed similar efficiency in NoV extraction from lettuce, suggesting that the ionic strength caused by NaCl in PBS is insufficient or is not the main factor for elution of viral particles.

Quantitative PCR was more sensitive than semi-nested PCR for detecting NoV GII in artificially contaminated, minimally processed lettuce. These results accord with results in other studies[26,27]. One possible explanation is that the polymerase activity may be more affected by inhibitory compounds co-extracted from lettuce than the Taq 5'exonuclease activity. The length of the amplicons obtained by qPCR (very short template) could also explain the best performance when using this method in relation to semi-nested PCR for amplifying the NoV GII polymerase region[27]. The use of 1:10 RNA dilution in the semi-nested PCR reduced the recovery success rates using GB and PBS. In this case, it is possible to assert that the use of RNA dilution, as a strategy to overcome inhibitors, reduced the target nucleic acid to a number that could not be amplified by the semi-nested PCR.

Conclusion

The substitution of PBS for GB for virus elution in the adsorption-elution method using negatively charged membranes, combined with the use of the filter bag, improved the method described previously by Fumian et al.[11]. Additionally, this study demonstrated the use PP7 as SPCV, thus revealing the method's feasibility for NoV recovery in food microbiology laboratories.

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