ORIGINAL ARTICLE

PCR, real-time PCR analysis on Norwalk virus in direct test on artificial-contaminated foodstuffs

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Abstract

Introduction The most commonly used methods to determine and identify Norwalk virus are based on molecular biology. *Methods* A viral extraction protocol from food samples was studied in this work using artificial contamination test. It consists of a new protocol with a phase of viral elution from the food matrix performed using an eluting solution (glycine and beef extract at 3% pH 9) and a concentration phase with polyethylene glycol 8000. To detect Noroviruses, two techniques of molecular biology, polymerase chain reaction and real-time polymerase chain reaction, were compared. At the same time, tests of direct viral identification were conducted on soft fruits and salad obtained from the market. *Results* From the results obtained it was possible to evaluate how the phase of viral recovery represents an important critical point of the protocol. *Conclusion* It was possible to identify a greater sensitivity of the real-time polymerase chain reaction compared with the traditional polymerase chain reaction.

Introduction

Noroviruses have a worldwide distribution, and they may be considered as the main agents of infectious gastroenteric illnesses in the industrialized countries, being responsible for 68–80% of gastroenteritis (Frankhauser *et al.*, 2002; Godoy *et al.*, 2006). The Centers for Disease Control and Prevention reckons that every year, there are at least 23 million cases of acute gastroenteritis due to this virus in the United States. For instance, in Minnesota alone, they proved to be responsible for almost the totality of the food-borne infections, having been identified as aetiological agents in 96% of the 90 viral epidemic foci (Deneen *et al.*, 2000).

One of the first major epidemics linked to water consumption took place in Finland (Kukkula, 1997) in April 1994, where about 25–50% of the population showed the typical symptoms of acute gastroenteritis. Laboratory research confirmed the presence of Adenovirus, Norwalk-like virus, small round viruses and Rotavirus belonging to groups A and C as infecting agents, but the more frequently implicated aetiological agent proved to be the Norwalk virus (NV). The deep aquifers located near the river were con-

taminated by the river water during spring floods (Kukkula, 1997).

European surveillance systems indicate that Noroviruses are responsible for about 50% of the cases of gastroenteritis reported in England and Wales. Similar data also come from Finland, Sweden, the Netherlands, Germany and Japan as evidence of their spreading worldwide (Lopman *et al.*, 2003; Kroneman, 2008). Several Norovirus epidemics were associated with the consumption of contaminated foods, chiefly shellfish such as mussels and oysters (Berg *et al.*, 2000) as well as the contamination of waters destined for human consumption (Butot *et al.*, 2007). Noroviruses have become an important public health problem worldwide due to their capacity to generate clinically remarkable infections in people of all age groups, their several modes of transmission as well as their highly genetic diversity, their low infecting dose (10–100 viral particles) and the inability of humans to develop a lasting immunity.

The aetiological agent responsible for most epidemics, due to the consumption of contaminated foods, is not often identified, although enteric viruses are deemed to be the main cause in most cases. The inability to confirming the

viral origin of these infections is often due to a lack of sensitive and reliable techniques for the viral identification in foodstuffs (Svenson, 2000).

A major problem is that two of the most important enteric viruses – such as NV and hepatitis A virus – do not grow on cell cultures, hence the implementation of a methodology allowing the detection of such viruses is very difficult.

Therefore, the standardization of a technique allowing the detection of the presence of such viruses in foodstuffs would prove to be essential.

Over the years, various methods have been applied to isolate and detect viruses in foodstuffs, notably in shellfish, waters, vegetables and fruits. The choice of the method depends on the evaluation of a number of analytical aspects that are mainly: the viral load, the food matrix, the determination times and costs. The most used methods, which are currently used to determine and identify the viruses are the *cell cultures*, the techniques of molecular biology [*probes*, *polymerase chain reaction* (*PCR*)] and the integrated systems (*cell cultures-PCR*). The past few years have witnessed the development of innovative methods which is being used more and more frequently: *real-time PCR*, which, besides offering all the advantages of the traditional PCR such as sensitivity, specificity and rapidity, offers also the advantage of monitoring the amplification reaction in real time. Real-time PCR gives quantitative results by detecting the accumulation of amplicons during the early PCR exponential phase.

The purpose of this study was to conceive and implement a methodology for detecting noncytopathogenic viruses from food samples through methods of molecular biology, and the application and optimization of a protocol in experimental test on artificial contamination to assess the percentage of viral recovery and to compare classic PCR with real-time PCR.

Materials and methods

Used noncytopathogenic viruses

NV recovered from a stool sample was used for the comparison between traditional PCR and real-time PCR for artificial contamination tests and for the positive control.

NV extraction from stool samples

One gram of faeces was suspended in phosphate-buffered saline 0.01 M, stirred on a Vortex mixer for about 60 sec,

centrifuged at 1000 r.p.m. (3354 g) and then filtered. The samples were then stored in the $-80\,^{\circ}C$ freezer.

Artificial contamination tests

Frozen strawberries and soft fruits from the market were chosen as food samples. To contaminate the foodstuff, 5 g of strawberries or soft fruits were immersed in 10 mL of 1/100 diluted viral suspension of NV and left in contact for 1 h. At the end of the contact time, the foodstuff were removed under aseptic conditions and put in contact for 20 min with 10 mL elution buffer containing 50 mM glycine, 100 mM Tris-HCl and 3% beef extract pH 9.2 and shaking at room temperature. The homogenate was centrifuged at 10 000 r.p.m. (3354 g) for 15 min, and the recovered elution buffer was adjusted to pH 7.2. At the end of the sequence, polyethylene glycol 8000 1:4 v/v was added to this suspension, and this was left in contact overnight at $4\,^{\circ}C$. The following day centrifugation at 10 000 r.p.m. (3354 g) at $4\,^{\circ}C$ was performed and the pellet was suspended in 2 mL of sterile water. Finally, after a further centrifugation at 10 000 r.p.m. (3354 g) for 15 min at $4\,^{\circ}C$, the aqueous phase was collected.

At each step of the protocol an aliquot was used for nucleic acid extraction, retrotranscription and amplification by real-time PCR.

Direct viral identification from samples of strawberries and soft fruits

The samples of strawberries and soft fruits to be used for the direct virological investigation underwent elution, homogenization and concentration according to the procedures
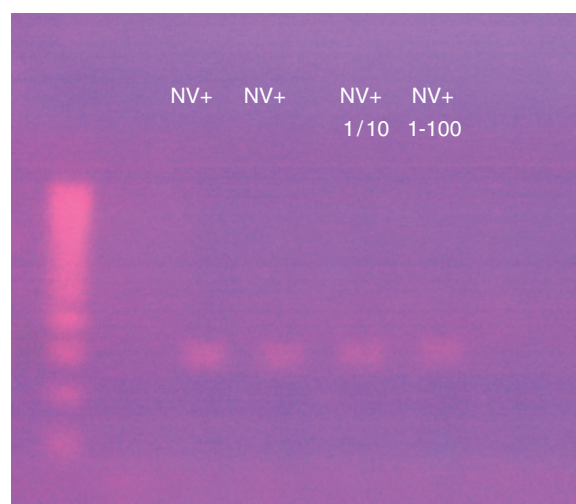
Figure 1 Traditional polymerase chain reaction electrophoretic run of Norovirus dilution stool sample.

described in the previous paragraph. The final suspension was used for nucleic acid extraction, retrotranscription and amplification by real-time PCR and PCR.

Viral RNA extraction

The extraction was carried out using a commercial kit 'Spin RNAII Macherey-Nagel Nucleus', sticking to the enclosed protocol. This procedure allows for the extraction, separa-

tion and purification of the viral RNA by eliminating the cell nucleic acids, DNases and any ribonucleases present.
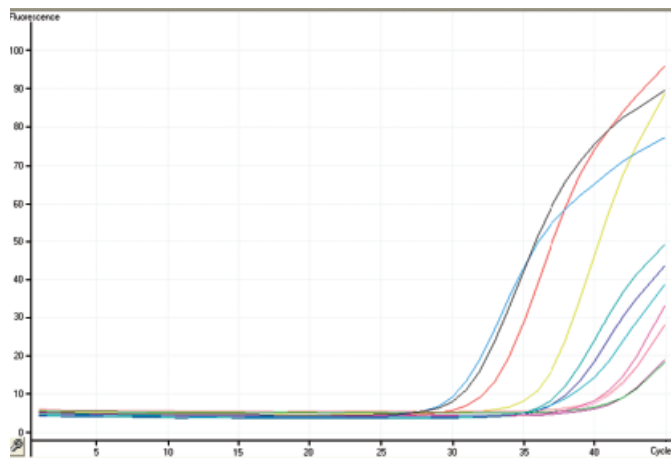
RNA retrotranscription

The search for the specific nucleotide sequence on RNA through the PCR includes viral RNA retrotranscription using a reverse transcriptase (MuLV reverse trasciptase 2.5 U/µL), $MgCl_2$ (5 mM), PCR buffer (1 ×), dNTPs (4 mM), RNase
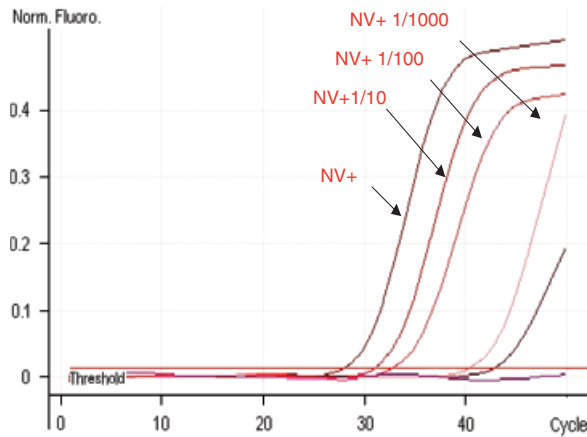
Figure 2 Real-time polymerase chain reaction of Norovirus dilution stool sample.
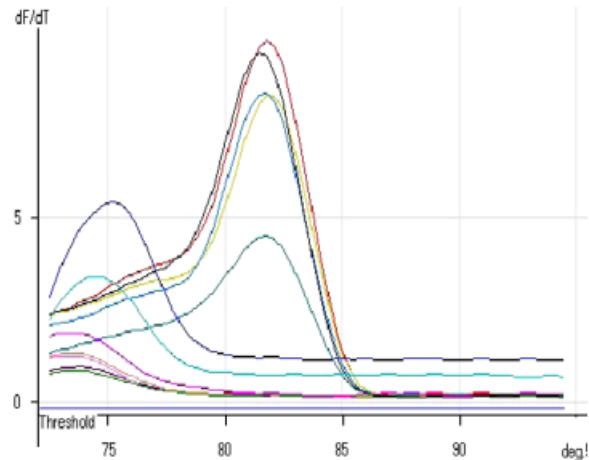
Figure 4 Melting curve. Melt data for Melt A.FAM/Sybr.

No.	Colour	Name	
1		Virus suspension A	
2		washing A	
3		eluated + c A	Test A
4		eluated − c A	
5		concentrated A	
6		Virus suspension B	
7		washing B	
8		eluated + c B	Test B
9		eluated − c B	
10		concentrated B	
11		Positive	
12		Real Time negative Cont	

Figure 3 Real-time polymerase chain reaction of the different steps of test A and test B.

inhibitor (1 U/µL), random Examer (2.5 U/µL) in order to obtain a complementary DNA (cDNA) acting as template for the following amplification phase.

NV amplification (Booster-PCR)

After obtaining the cDNA from viral RNA, the considered region was amplified by carrying out two consecutive PCRs with the same primer pair shown in the table below.

Primer	Sequence (5′–3′)
GII reverse	5′ CC(AG) CC (AGCT) GCA T(AG) (ATC) CC(AG) TT(AG) TAC AT 3′
GII forward	5′ C (AGCT)T GGG AGG GCG ATC GCA A 3′

Real-time PCR on Norovirus

A real-time PCR was carried out using a Mastermix 2X Power SYBR Green (Applied Biosystem, Foster City, CA, USA), which includes a final volume of 20 µL of which

● 10 µL of Master mix SYBR Green containing: SYBR Green 1 Dye, AmpliTaq Gold DNA Polymerase, dNTPs with dUTP;

● 10 µL of mixture consisting of 5 µL of cDNA and 5 µL ofprimers in RNase free H$_2$O.

The used primers are the following:
COG2R: 5′ TCG ACG CCA TCT TCA TTC ACA 3′
QNIF2d: 5′ ATG TTC AGR TGG ATG AGR TTC TCW GA 3′

Results and discussion

Comparison between traditional PCR and real-time PCR in Norovirus-positive samples

The extract from a faecal sample positive for NV was amplified at various dilutions with traditional PCR. The electrophoretic run detected a positivity for NV dilution 1/10 and 1/100 (Figure 1). The same sample at different dilutions was also amplified with real-time PCR, and in this case a positivity for the dilution 1/1000 (Figure 2) was also detected. These data highlighted a greater sensitivity of the real-time PCR compared with the traditional method.
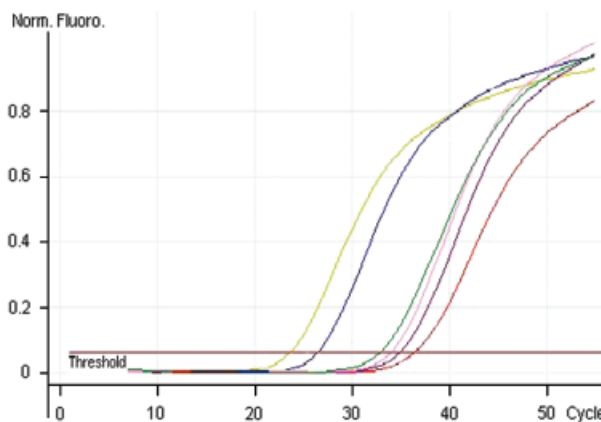
Artificial contamination

Two tests A and B, of artificial contamination carried out with Norwalk virus and analysed by using real-time PCR
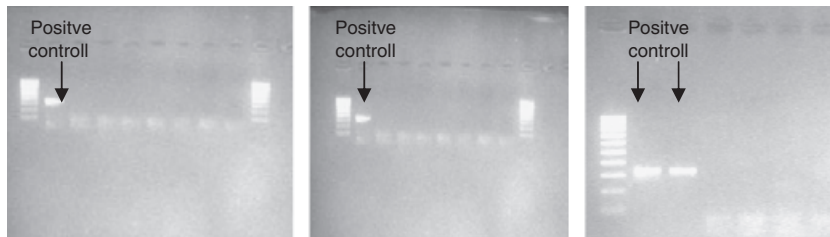
Figure 5   Some electrophoretic runs of direct viral identification analyses in food samples.

No.	Colour	Name	Ct
1		salad	36,72
2		I positive cont.	23,87
3		II positive cont.	26,69
4		Negative cont.	35,13
5		RT negative cont.	34,19
6		Real Time negative Cont,	33,06

Figure 6   Results of Real-time polymerase chain reaction.

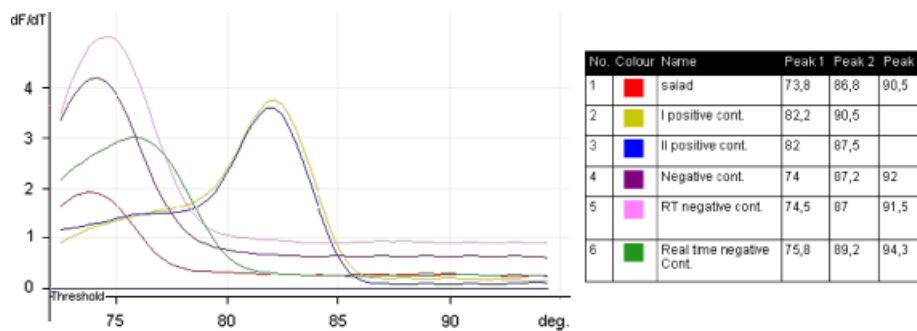| No.	Colour	Name	Peak 1	Peak 2	Peak 3
1		salad	73,8	86,8	90,5
2		I positive cont.	82,2	90,5	
3		II positive cont.	82	87,5	
4		Negative cont.	74	87,2	92
5		RT negative cont.	74,5	87	91,5
6		Real time negative Cont.	75,8	89,2	94,3

Figure 7 Melting curve.

detected the positivity of the starting sample and the sample corresponding to the washing phase for both tests only, whereas in the samples corresponding to the elution and concentration phase no positivity was found. The result obtained through the real-time PCR is reported in Figure 3 and it is possible to see fluorescence increasing with the different samples analysed in terms of cycle numbers. On the other hand, in Figure 4 it is possible to see the melting temperature and the overlapping of temperature peaks overlap for the tested samples compared with the one obtained with positive control.

Tests of direct viral identification

Traditional real-time PCR

Previously tests of direct viral identification of NV from food matrixes (samples of frozen soft fruits found on the market belonging to different brands) did not detect the presence of the virus. Figure 5 shows the result obtained from real-time PCR tests carried out on fruits and vegetables for the detection of NV.

Real-time PCR

A test of direct identification of NV in a salad sample was carried out through real-time PCR. The results obtained highlighted the two positive samples, whereas no positivity was found in the salad sample. In Figure 6 fluorescence graphic in terms of the function of cycle numbers is shown and in Figure 7 it is possible to see melting temperature graphic. In the latter it is possible to see two overlapping peaks, which correspond to the positive controls.

Conclusions

Our data showed positive results regarding the sensitivity of real-time PCR and resulted in better detection than ob-

tained with the traditional PCR for Norovirus. The tests with artificial contamination detected the Norovirus in the washing sample only, whereas it was detected neither in the eluate nor in the concentrate in confirmation of the recovery difficulties – as already been pointed out during one of our previous studies using cytopathogenic viruses, and in other studies (Katayama *et al.*, 2002; Butot *et al.*, 2007; Zoni *et al.*, 2009) The phase of detachment of viral particles from the substrate seems still to be the most delicate passage for the Norovirus among the various phases. The study will go on with the artificial contamination tests to evaluate the suitable changes of the various phases to optimize the method.

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