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ORIGINAL PAPER

# Study on the Application of a New Method for the Viral Detection of *Caliciviruses* in Foodstuffs

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## Abstract

*Noroviruses (NoVs), transmitted mainly through the fecal-oral route, are particularly infectious and only a few virions carried by contaminated water or food are sufficient to cause the disease. These viruses may cause outbreaks of large proportions that could be limited if there is a quickly detection of the source of infection and if would be prohibited or restricted to the sick people the contact with others. Foods normally involved in this disease are edible bivalve molluscs (mussels, oysters and clams) that are often eaten raw or under-cooked and frozen soft fruits imported from areas with low hygienic conditions. We developed a rapid, reliable and sensitive method for the viral detection in foodstuffs using proteinase K, a serine protease, often used in molecular biology to digest proteins and remove contamination from nucleic acids preparations. We applied this method to clams infected with Feline Calicivirus (FCV) strain F9 (used as substitute of NoV, which isn't able to replicate in vitro) and to soft fruits infected with a NoV solution. The protocol consists of three steps: first we homogenized the foodstuffs, then we put the samples in a tube together with proteinase K for an hour at 37°C and finally we centrifuged the sample at 4300 rpm for 5 min. We noticed a cytotoxic effect of proteinase K on cell cultures for tests with FCV and to avoid the problem we will add a phase of inactivation of the enzyme with a proper inhibitor. For tests with NoV we used for the detection the Real-time PCR that allowed also a quantification of the samples. Coupling our protocol with Real-time PCR makes detection quicker and it was possible to detect even low concentrations of viruses. Besides a step with RNAse A will be introduced together with proteinase K to avoid false positive with real-time PCR.*

**Keywords:** Norovirus, gastroenteritis, proteinase K, Real-time PCR.

## Introduction

Norwalk virus was identified as the principal agent of an acute gastroenteritis outbreak occurred in 1968 in an elementary school in Norwalk, Ohio, during which nearly half of the students and a third of the personnel contracted the infection. Lately the agent was recovered in many other acute gastroenteritis outbreaks and in 1972 the virus was identified in stool samples by electron microscopy and was added to the *Caliciviridae* family. Noroviruses (NoVs) are the leading cause of acute nonbacterial gastroenteritis resulting in 23000000 infections, 50000 hospitalizations and 300 deaths per year only in the U.S.A. Although the course of the illness is mild and self-limiting, infants, elderly and immunocompromised patients may develop serious complications caused by vomiting and protracted diarrhoea. These viruses are transmitted by faecal-oral route and the main sources of infection are water [1], faecally contaminated foodstuffs (prepared by infected foodhandlers), contaminated surfaces and direct person-to-person contact which is the main mode of transmission in health institutions. Foods normally involved in this disease are edible bivalve molluscs (mussels, oysters and clams) that are often eaten raw or under-cooked [2], and frozen soft fruits imported from areas with low hygienic controls [3]. NoVs outbreaks occur mainly in

communities, nursing homes, cruise ships, schools, hospitals, restaurants and army barracks. This viral agent causes acute gastroenteritis all the year but there is a higher incidence during the winter months.

These viruses may cause outbreaks of large proportions that could be limited if there is a quickly detection of the source of infection and if would be prohibited or restricted to the sick people the contact with others. Data provided by the UK Health Protection Agency reported that in a month 45 outbreaks of gastroenteritis occurred in hospitals, 38 were confirmed by laboratories as due to Norovirus and 25 of these have led to ward closure [4]. It is important to remind that NoVs are a threat not only for the public health but also under an aspect purely economic in terms of costs for the sanification of patient's room as well as the costs for cares provided to the sick and also in terms of working hours and wages lost by ill people.

Taking into consideration NoVs characteristics we tried to develop a rapid, reliable and sensitive method that allows the detection of these viruses in foodstuffs using proteinase K, a serine protease, often used in molecular biology to digest proteins and remove contamination from nucleic acids preparations [5]. As is often reported in literature NoVs do not replicate *in vitro* (or is very difficult to do so), for this reason it is necessary to use substitutes such as Feline Calicivirus (FCV) to perform experimental tests [6]. As a result, the principal technique for the detection of NoVs has always been the PCR in particular in its Real-time mode that allows having a quantity and quality data.

## Materials and Methods

### VIRAL SUSPENSION PREPARATION AND FOODSTUFFS USED

We carried out artificial contamination tests on foodstuffs using the following viruses: FCV strain F9 (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, IZSLER) and NoV genogroup II obtained from stool samples of infected patients. We suspended 1 g of faeces in 9 ml of 0.01 M Phosphate buffered saline (PBS). This suspension was shaked with vortex for 60 s, centrifuged at 10000 rpm and then filtered using 0.22 μm diameter-pore filters to remove bacterial contaminations. The viral solutions thus obtained were stocked at -80°C.

FCV viral suspension was obtained inoculating 0.1 ml of virus directly on the cell layer (Crandell Reese Feline Kidney cells) in a cell culture flask deprived of growth medium and washed twice with PBS. The flask was incubated in thermostat at 37°C for 1 h, at the end of which the growth medium was newly added and the flask was replaced in the thermostat at 37°C until we obtained the maximum cytopathic effect. Then the flask was subjected to 3 cycles of freeze-thaw at -20°C after which the culture medium was centrifuged at 2000 rpm for 2 min to separate the surnatant containing the virus. The viral suspension was then purified by centrifugation using Amicon Ultra-15 Centrifugal Filter Units (Millipore, Billerica, MA, U.S.A.) at 4300 rpm at 4°C. Virus retained on the filter unit membrane was suspended with sterile saline solution and stocked at -80°C.

Food samples were found on the market and they are: clams coming from the Po river delta and frozen soft fruits.

### PROTEINASE K PROTOCOL

The protocol involves an initial homogenization of the samples; 2 g of the homogenate are placed in a tube with 2 ml of a proteinase K solution (Novagen, Madison, U. S. A). Proteinase K solution is prepared from one with a starting concentration of 600 mAnson-U/ml, bringing the concentration to 3 mAnson-U/ml. One unit is defined as the amount of enzyme that will liberate 1.0 μmol tyrosine from casein per min at 37°C, pH 7.5. Tubes are left continuously shaking for 60 min at 37 °C and then the samples are centrifuged at 4300

rpm for 5 min and the supernatant is recovered for the successive phases of analysis and titration.

**SOFT FRUITS TESTS**

Soft fruits were weighed and placed into beaker; tests were conducted with 70, 30 and 10 g of food and a NoV solution, diluted 1000 times from a stool sample, has also been prepared. Tests consist in vaporising the solution with a spray on the food samples and then we homogenate the soft fruits and we take 2 g of sample on which we apply the proteinase K protocol. RNA was extracted using Nucleo Spin RNAII kit (Macherey-Nagel, Düren, Germany) sticking to the enclosed protocol and then the nucleic acids were retrotranscripted into cDNA using the reagents and thermal profile shown in table 1 and table 2.

**Table 1** Reagents used for retrotranscription step.

| Reagents (Initial conc.) | Final Conc. | Quantity per sample |
|---|---|---|
| dNTPs (10mM) | 4mM | 8µl |
| $MgCl_2$ (25mM) | 5mM | 4µl |
| PCR buffer (10X) | 1X | 2µl |
| Random hexamer (50µM) | 2,5µM | 1µl |
| RNAsi inhibitor (20U/µl) | 1U/µl | 1µl |
| MuLV reverse transcriptase (50U/µl) | 2,5U/µl | 1µl |
| RNA | | 3µl |

**Table 2** Thermal profile used for retrotranscription step.

| Fase | Temperature | Time | Cycles |
|---|---|---|---|
| Retrotrascription | 42°C | 60 min | 1 |
| Denaturation | 94°C | 5 min | 1 |

The cDNA was then used as template for the Real-time PCR where in every tube, with a final volume of 20 µl, was present:

- 10 µl of Power SYBR Green Master mix (Applied Biosystem**,** Life Technologies Corporation**,** Carlsbad, California, U.S.A.) containing: SYBR Green 1 Dye, AmpliTaq Gold DNA Polymerase, Deoxynucleotide-Triphospates (dNTPs);
- 5 µl of primers [7] and RNAse free water;
- 5 µl of cDNA.

**Table 3** Primers sequence used for real-time PCR.

| Primers | Sequence |
|---|---|
| COG2R | TCGACGCCATCTTCATTCACA |
| QNIF2d | ATGTTCAGGTGGATGAGATTCTCTGA |

**Table 4** Thermal profile for real-time PCR.

| Fase | Temperature | Times | Cycles |
|---|---|---|---|
| Denaturation | 95°C | 5 min | 1 |
| Denaturation | 95°C | 15 s | 45 |
| Annealing | 60°C | 60 s | 45 |
| Elongation | 72°C | 60 s | 45 |
| Cooling | 40°C | 60 s | 1 |

The amplification, detection and data analysis was carried out with Rotor Gene 6000 (Corbett Life Science, San Francisco, California).

**CLAMS TESTS**

We prepared an aquarium with 15 litres of 3% saline water maintained at 14°C-17°C where about 1 Kg of clams remained for at least 24 h; then 25 ml of a solution containing FCV with an unknown titre was poured in the aquarium water. We recovered the clams on the day after the contamination, we shelled them and we recovered only the hepatopancreas which is the organ where everything filtered by the clam is concentrated. The hepatopancreas are homogenized in a beaker and 2 g of this homogenate are placed in a tube with 2 ml of proteinase K solution and subjected to the protocol as described previously. At the end of the protocol we recovered the supernatant for every test and from these samples we set up scalar 10-fold dilutions. We also prepared 96-wells plates with 25 µl per well of Minimum Essential Medium (MEM) with Earle's salt and L-Glutamine (PAA Laboratories GmbH, Pasching, Austria) then we inoculated the dilutions (25µl per well and 16 wells per dilution). Finally we added 50 µl of cell suspension (Crandell Reese Feline Kidney cells) for each well. The plates were incubated at 37°C with 5% of $CO_2$ and after 6 days we detected the presence of the cytopathic effect induced by the virus. The cytopathic effect is visible on the cell layer and we have calculated for every dilution the number of positive wells (the ones with an evident cytopathic effect). The viral titre was calculated according to the Reed-Müench method which is a 50 percent endpoint assay. This method involves the determination of the dilution of inoculum producing 50 percent infection of the wells. We have to calculate an index with the formula provided by Reed-Müench showed in Eq. (1)

$$Index = \frac{A - 50}{A - B} \tag{1}$$

where A is the percentual of infected wells at the dilution immediately above 50% and B is the percentual of infected wells at the dilution immediately below 50%.

This index is applied to the dilution that produced the infection rate immediately above 50 percent and the reciprocal of this result is the value of $TCID_{50}$ in 25 µl. Then we multiply this value by four to have the $TCID_{50}$ per 100 µl which is the total volume of the solution contained in one well. Finally we express our results as the logarithm of $TCID_{50}$ to normalize the data collected.

## Results and Discussions

**SOFT FRUITS TESTS**

First of all a calibration curve was set for the real-time machine to allow the quantification of the samples. Then we carried out real-time PCRs on soft fruits samples; for every test a different number of sprays was vaporised on the samples. Every spray has a volume of about 110 µl and the NoV starting solution has a concentration of 368 virions/ µl. The results are shown in table 5.

**Table 5** Results of tests with soft fruits, for positive samples the viral concentration is indicated.

| Soft fruits (g) | Sprays | Result | Concentration (copies/µl) | Soft fruits (g) | Sprays | Result | Concentration (copies/µl) |
|---|---|---|---|---|---|---|---|
| 70 | 10 | negative | | 30 | 4 | negative | |
| 70 | 9 | negative | | 30 | 3 | negative | |
| 30 | 10 | positive | 2,368085324E+01 | 30 | 2 | negative | |
| 30 | 9 | negative | | 30 | 1 | negative | |
| 30 | 8 | positive | 4,425171295E+00 | 10 | 10 | negative | |
| 30 | 7 | positive | 6,019548229E+01 | 10 | 9 | negative | |
| 30 | 6 | positive | 2,678964419E+01 | 10 | 8 | negative | |
| 30 | 5 | negative | | 10 | 7 | negative | |

In the table we observe that with 70 g of soft fruits we cannot obtain detectable results with real-time PCR; this data can be explained with the great amount of foodstuff compared to the virus present in the beaker. With 30 g of soft fruits, instead, we collected good results for tests with a high number of sprays but in tests with 5 to 1 sprays we did not collect any result probably for the same reason of tests with 70 g. We also had no results with 10 g of foodstuff by now. In table 5 we observe that we have a negative result with 9 sprays for the 30 g soft fruits sample and this is probably due to an error in one of the steps of the protocol. We have to take into consideration also that we tried to reproduce every spray exactly as it was the precedent but we wasn't able to control if it was so. This implies that for some samples the amount of virus sprayed could vary. At the beginning we decided to spray the viral solution on the foodstuffs to simulate the natural way the soft fruits are contaminated. The results obtained show that our method works well and is sensitive enough to detect low concentration of virus but to make sure that the soft fruits will be contaminated with a sufficient amount of virus and to eliminate the variability between the samples we will immerse the foodstuffs into a viral solution with an equal concentration for every sample.

**CLAMS TESTS**

For tests with clams we assessed the viral titre in contaminated water expressed as the logarithm of $TCID_{50}$ that was 4.37 while in the solution used to contaminate the water was an average of 7.72. Initial tests, carried out by taking the whole clam body, have given negative results for this reason we decided to recover only hepatopancreas. We noticed a cytotoxic effect in cell culture due to proteinase K so we could not display the results of the clam tests. We have inoculated only the enzyme with cell cultures and we obtained the same cytopathic effect; for this reason we added a new step in the protocol with a proteinase K inhibitor: Methoxysuccinyl-Alanine-Alanine-Proline-Phenylalanine-Chloromethyl Ketone (MeOSuc-AAPF-CMK). This substance, as well as other similar to it, has been described as an effective proteinase K inhibitor [8]. Tests conducted with this new protocol have given good results and we noticed that concentrations of 0.5, 0.1, 0.05, 0.01 mM are sufficient to inactivate the enzyme.

## Conclusions

We noticed from our results that the protocol with proteinase K is fast and, in association with real-time PCR, also rather sensitive. In fact every sample is subjected to a step of purification with proteinase K that last 1 h and then a centrifugation for 5 min. The samples after these two passages are ready for biomolecular analysis such as RT and real-time PCR that last together only few hours. Ultimately altogether these analyses provide results in one day unlike other protocols we used which involves the use of elution buffers and Polyethylene Glycol (PEG) with a molecular weight of 8000 g/mol to purificate and concentrate the viral particles [9]. In this previous work we used a two-day protocol that provides a first step of detachment of the viral particles from the food sample involving an elution buffer containing glycine, Tris-HCl and beef extract followed by a centrifugation and finally a contact overnight with $PEG_{8000}$. The second day after another centrifugation the pellet was suspended with 2 ml of sterile water and the samples were tested with classic and real-time PCR with the same profile we used in this work. The results obtained with the proteinase K protocol are better than that obtained in our previous work regarding the recovery rate and speed of treatment of the samples. We can also appreciate the sensitivity of the method that coupled with real-time PCR detects even few viral particles per μl.

It will be necessary to perform other tests to confirm the data collected and subsequently vary the parameters such as the initial concentration of the viral suspension. Besides we could add in the protocol a treatment with RNAse A to restrict the presence of false positive in real-time PCR [10, 11, 12].

The problems linked to the cytopathic effect on cell cultures due to proteinase K do not seem to affect the results of PCR tests probably because the soft fruits samples are subjected to a RNA extraction step that cleans the samples and also because the proteinase K is not active on nucleic acids. To use this protocol with the type of virus that grows on cell cultures we added a step with an inhibitor of the enzyme at the concentration of 0.05 mM. Since the first results we have obtained with this inhibitor were positive then we think that this shrewdness is enough to avoid the cytopathic effect of the enzyme on cell cultures.

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