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A comparison of proteinase K and PEG_{8000} on the recovery of calicivirus and norovirus in artificially contaminated food

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RESEARCH ARTICLE

Abstract

Noroviruses (NoV), transmitted mainly through the faecal-oral route, are particularly infectious and only a few particles carried by contaminated water or food are needed to cause the disease. In particular raspberries are often the cause of the onset of an outbreak. These viruses may cause large-scale outbreaks that could be limited if there was a rapid detection of the source of infection. The aim of this study is to compare the results of a method for viral detection in foodstuffs using proteinase K, a serine protease, with one that uses PEG_{8000} in order to assess which one provides the highest viral recovery. Mixed berries were artificially contaminated with a viral solution – feline calicivirus (FCV) or NoV – and they were analysed with the two methods for the elution of viral particles. In tests with FCV the method with proteinase K was able to obtain recovery percentages on average about three times higher than with PEG_{8000} (68.297 vs. 23.989). The difference between the two recoveries is statistically significant, whereas in tests with NoV, even if the protocol with proteinase K provided the best results the gap between the two methods isn't statistically significant. The proteinase K protocol obtained better recoveries in tests with both FCV and NoV. This method has the potential to become the official standard technique for the detection of viruses in food matrices.

Keywords: viral recovery, gastroenteritis, real-time PCR

1. Introduction

Norwalk virus was identified as the principal agent of an acute gastroenteritis outbreak that occurred in 1968 in an elementary school in Norwalk, Ohio, affecting nearly half of the students and a third of the personnel. Later the agent was recovered in many other acute gastroenteritis outbreaks and in 1972 the virus was identified in stool samples by electron microscopy (Kapikian *et al.*, 1972) and added to the *Caliciviridae* family. Noroviruses (NoV) are the leading cause of acute nonbacterial gastroenteritis resulting in 23 million infections, 50 thousand hospitalisations and 300 deaths every year in the USA alone (Mead *et al.*, 1999). This is a heterogeneous group divided into five genogroups: genogroups I, II and IV infects humans while the other two contain murine and bovine strains. The virus found during the outbreak in Ohio has become the type species of the Noroviruses and is included in genogroup I. The principal symptoms are those associated with viral gastroenteritis: vomiting, diarrhoea, nausea, abdominal pain and mild fever. Although the course of the illness is mild and self-limiting, infants, the elderly and immunocompromised patients may develop serious complications caused by vomiting and protracted diarrhoea. These viruses are transmitted by the faecal-oral route and the main sources of infection are water (Pedalino *et al.*, 2003), faecally-contaminated foodstuffs (prepared by infected food handlers), contaminated surfaces and direct person-to-person contact which is the main mode of transmission in health institutions. An important route in this last mode of transmission is the spread of infected droplets among individuals; particularly droplets of vomit that could reach healthy people in the vicinity of sick people. Foods normally involved in this disease are edible bivalve molluscs (mussels, oysters and clams) that are often eaten raw or under-cooked (Alfano-Sobsey *et al.*, 2011), batches of frozen strawberries or raspberries

imported from areas with poor hygiene controls (Cotterelle *et al.*, 2005; Korsager *et al.*, 2005; Maunula *et al.*, 2009; Sarvikivi *et al.*, 2011) and lettuce (Ethelberg *et al.*, 2010). In Germany the largest foodborne outbreak of gastroenteritis which caused 11 thousand cases among young people was linked to the consumption of a dessert made with frozen strawberries. Berries are often linked to outbreaks of NoV due to their particular conformation; those fruits have often a bunch configuration made of drupelets or an indented peel. This property allows the viruses to attach to the peel where they can remain hidden and out of reach even if the food is thoroughly washed. NoV outbreaks occur mainly in communities, nursing homes, cruise ships, schools, hospitals, restaurants and army barracks. This viral agent infects people of all ages and causes acute gastroenteritis all the year round, though there is a higher incidence during the winter months.

These viruses may cause large-scale outbreaks that could be limited if there is a rapid detection of the source of infection, if sick patients are isolated from non-infected people and if there is a strategy in place for implementing hand hygiene and contact precautions (Gilbride *et al.*, 2009, Johnston *et al.*, 2007). Data provided by the UK Health Protection Agency reported that in 2011, of all the outbreaks in healthcare settings, 65% were confirmed by laboratories as due to Norovirus (http://tinyurl.com/nrnkg96). It is important to remember that NoV are a threat not only for public health but also financially in terms of the costs for cleaning patients' rooms as well as costs for care provided to the sick and in terms of working hours and wages lost by patients. The cost of outbreaks in the UK has been estimated at 1 million pounds per 1,000 hospital beds (Lopman *et al.*, 2004). NoV is highly infectious and less than 100 virions are required to cause the disease; it is also resistant to disinfectant activity when this is not preceded by a detergent when cleaning surfaces. On this basis it is important to prevent the contamination of surfaces, food and the spread of NoV via person-to-person contact by implementing the correct hygiene practices and disinfection (Said *et al.*, 2008). Taking into consideration NoV characteristics, it is extremely important to develop rapid, reliable and sensitive methods which allow the detection of these viruses in foodstuffs. The aim of this study is to develop a method to detect caliciviruses in food samples involving proteinase K, a serine protease often used in molecular biology to digest proteins and remove contamination from nucleic acids preparations (Costantini *et al.*, 2006). To assess the efficacy of this method the results obtained are compared with another protocol which involves the use of PEG_{8000} in order to see which one is the best. As is often reported in the literature, NoV do not replicate *in vitro* thus the technique of election for their detection has always been the polymerase chain reaction (PCR) in particular in its real-time mode that allows having a quantity and quality result. That is the reason why, together with the fact that PCR does not discriminate between infectious and non-infectious viruses, it is necessary to use substitutes such as feline calicivirus (FCV) to perform experimental tests which require the use of cell cultures (Cannon *et al.*, 2006; Richards, 2012).

2. Materials and methods

Viruses and cell culture used

For this study the following viruses were used: FCV strain F9 (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, IZSLER) and NoV genogroup II obtained from stool samples of infected patients. Stool samples were clarified and 1 g of faeces was placed in a tube with 9 ml of 0.01 M phosphate buffered saline (PBS); then the tube was shaken with vortex for 60 s, centrifuged at 10,000×*g* for 10 min and then filtered using 0.22 µm diameter-pore filters to remove bacterial contaminations. The viral solution thus obtained was 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 at 37 °C for 1 h, then the growth medium was added and the flask was incubated again at 37 °C until the appearance of the maximum cytopathic effect. The flask was stored at -20 °C until its use when it was subjected to 3 cycles of freeze-thaw after which the culture medium was centrifuged at 1000×*g* for 2 min to separate the supernatant containing the virus. The viral suspension was then purified by centrifugation using Amicon Ultra-15 Centrifugal Filter Units (Millipore, Billerica, MA, USA) at 4,000×*g* at 4 °C. The filter membrane was rinsed with sterile saline solution that is recovered and stocked at -80 °C.

The study consisted of the assessment of the recovery rate of two different methods applied to food artificially contaminated with caliciviruses. The food samples used were retrieved on the market and they were a mix of frozen berries containing raspberries, blueberries, wild blackberries and redcurrants. The samples were thawed before the tests.

Proteinase K protocol

The protocol involves an initial homogenisation of the contaminated samples; 2 g of the homogenate are placed in a tube with 2 ml of a proteinase K solution at a concentration of 0.1 mg/l (Novagen, Madison, WI, USA). Tubes are left continuously shaking at 150 rpm for 60 min at 37 °C and then the samples are centrifuged at 4,000×*g* for 10 min at 4 °C and the supernatant is recovered for the successive phases of analysis and titration.

The first tests revealed that proteinase K produced a cytotoxic effect in cell cultures and for this reason a step with an inhibitor of the enzyme was added for tests with FCV. The inhibitor is methoxysuccinyl-alanine-alanine-proline-phenylalanine-chloromethyl ketone (MeOSuc-AAPF-CMK) (Life Technologies, Monza, Italy); 10 µl of this compound were added to the supernatant reaching the final concentration of 0.05 mM. This substance, as well as others similar to it, has been described as an effective proteinase K inhibitor (Anilkumar *et al.*, 2009). Tubes were shaken and left for 15 min at room temperature to allow the inactivation of the enzyme; then the samples were centrifuged again at 4,000×*g* for 10 min at 4 °C. To the recovered supernatant was added 0.1 ml of a mix of antibodies containing penicillin, streptomycin and ampicillin. The antibodies were added to prevent bacterial contamination when analysing the titres in cell cultures.

PEG_{8000} protocol

This protocol lasts three days and is divided into several passages of centrifugation, cleaning and concentration of the sample using polyethylene glycol with a molecular weight of 8,000 Da (PEG_{8000}).

The first day the contaminated berries were homogenated in a beaker with a mixer and the glycine buffer was added at a ratio of 1:1. The beaker was left at 4 °C for 30 min and was shaken every 5 min to permit the mixing between the buffer and the homogenate. The solution was recovered in tubes and was centrifuged at 10,000×*g* for 20 min at 4 °C; then PEG_{8000} was added to the supernatant at a ratio of 1:4. The tubes were shaken and left overnight at 4 °C.

The second day the tubes were centrifuged at 10,000×*g* for 45 min at 4 °C; the supernatant was eliminated and 10 ml of sterile water was added to suspend the pellet. Then another centrifugation was performed at 10,000×*g* for 20 min at 4 °C to clean the samples and PEG_{8000} was added at the same ratio as the previous day. Tubes remained overnight at 4 °C.

The third day the centrifugations are performed with the same parameters as the second day, although only 2 ml of sterile water was used to suspend the pellet. After the last centrifugation the supernatant was recovered and a mix of antibodies was added.

Tests with feline calicivirus

A viral suspension containing FCV was prepared and stocked at -80 °C until its use. The viral titre was determined and the suspension was diluted 100 times to obtain the desired concentration. The solution was divided into several tubes containing 42 ml each and stocked at -20 °C till their use.

A beaker was weighed empty and then 30 g of the berries mix was added. Once thawed, 10 ml of the solution was poured on the foodstuff and left in contact for 30 min to contaminate the sample. Afterwards all the liquid was recovered, classified as 'recovered solution' and analysed to calculate the viral titre. The berries left in the beaker were mixed and 2 g of the homogenate was placed in a tube with proteinase K as described previously. Meanwhile the rest was weighed and glycine buffer was added following the PEG_{8000} protocol.

A total of 18 tests were performed. Since the contaminating solution was stocked in batches of 42 ml, to avoid a decrease in the viral titre it was decided to use all the solution once thawed. For every tube, three different beakers were contaminated with 10 ml of the solution and analysed with the two protocols. Another 10 ml of the solution was placed in an empty beaker to be used as a positive control whereas the remaining 2 ml was analysed to calculate the initial viral titre of the contaminating solution.

Once all the samples were obtained, the titre was calculated according to the Reed-Muench method (Reed and Muench, 1938). Briefly the method consists of counting wells in which there is an evident cytopathic effect to assess where 50% of the infected cell culture is situated. In this way the $TCID_{50}$ (50% tissue culture infective dose) is calculated and the logarithm of this value is obtained to normalise all data.

Data obtained were analysed further with the statistical software SPSS (IBM Corporation, Armonk, NY, USA) applying the Student's t-test with a confidence limit of 5% to investigate if there is a statistically significant difference between the recoveries of the two protocols.

Tests with human norovirus

Tests conducted using NoV were performed similarly to those involving FCV. The starting solution was thawed and diluted 10 times to obtain a suspension with which 30 g of berries was contaminated and left at room temperature for 30 min. Once all the liquid had been recovered, the remaining food was homogenised with a mixer. The proteinase K protocol was used for 2 g of homogenate, while the rest of the berries were analysed according to the other protocol.

In this case only six tests were performed because it wasn't possible to obtain further stool samples and the other samples stocked for more than two years had a starting titre that was too low to be useful in these tests.

Once the samples were obtained, the RNA was extracted using Nucleo Spin RNAII kit (Macherey-Nagel, Düren, Germany) conforming to the enclosed protocol, and then the nucleic acids were retrotranscripted into cDNA

using random hexamers and MuLV reverse transcriptase. The thermal profile is constituted in two steps: a retrotranscription at 42 °C for 60 min and a denaturation at 94 °C for 5 min. The cDNA obtained was used as a template for the real-time PCR. The primers COG 2R and QNIF2d (Mattison *et al.*, 2011) were added to the Power SYBR Green Master mix (Applied Biosystem, Life Technologies Corporation, Carlsbad, CA, USA) and after an initial denaturation at 95 °C for 5 min 45 cycles followed. In every cycle there were: 15 s of denaturation at 95 °C, 60 s of annealing at 60 °C and 60 s of elongation at 72 °C. The primers used in this study are directed to a small region of 88 bases which is a portion between ORF1 and 2 of the NoV genome. The amplification, detection and data analysis was carried out with a Rotor Gene 6000 (Corbett Life Science, San Francisco, CA, USA).

The quantification of the NoV samples was made possible after the set-up of a calibration curve. A sample of highly concentrated and purified segments of cDNA target was analysed with a Nano-drop 1000 UV (Thermo Scientific, Waltham, MA, USA), a spectrophotometer, to obtain the exact amount of RNA expressed in ng/µl. This value was used to calculate the number of molecules of RNA per µl. Then scalar ten-fold dilutions were set up and analysed in quadruplicate with Rotor Gene 6000. Once the analysis was performed, the software was able to design the calibration curve with an R value of 0.99. Even though the data set was not very large, it was decided to apply a Student's t-test setting the confidence limit to 5%.

3. Results and discussion

Tests with feline calicivirus

The determination of the viral titre in tests with FCV was achieved following the Reed-Muench method, and data were expressed as the logarithm of $TCID_{50}$ thus providing an absolute measure of the titre. In Table 1 the results are reported for every sample.

The starting solution has a mean titre of 4.668 whereas the solution recovered right before the homogenisation, which was analysed too, has a viral titre of 1.725. From these two values it is possible to assess the amount of virus remaining in berries by making a subtraction which results in a titre of 2.943. The food samples contaminated and subjected to each one of the two protocols to recover the viral particles provided different results. The protocol involving proteinase K gives a mean value of 2.01 while the other protocol resulted in a recovery of 0.706. There is a great difference between these two values and the data were analysed further using the statistical software SPSS with a Student's t-test. The test resulted in a statistically significant difference between the two values with $P<0.001$. In Figure 1, the results are presented graphically and clearly show the difference between the two methods.

The data obtained allow the determination of the recovery rates which are 68.297% for proteinase K protocol and 23.989% for PEG_{8000} protocol.

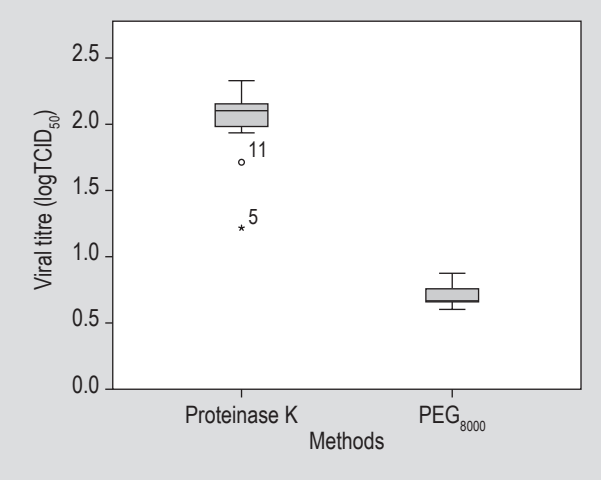

Figure 1. Graphic representation of values obtained with box plots.

Table 1. Values expressed as $LogTCID_{50}$ of all samples collected from tests with feline calicivirus.

Sample	Minimum	Maximum	Mean	Standard deviation
Starting solution	3.892	5.031	4.668	0.523
Recovered solution	1.012	2.216	1.725	0.400
PSPK[1]	0.675	1.269	0.982	0.332
PSPEG[2]	2.055	3.173	2.769	0.492
Proteinase K	1.217	2.329	2.010	0.292
PEG_{8000}	0.602	0.875	0.706	0.080

[1] PSPK is the value which belongs to the positive control solution to which the proteinase K protocol was applied.
[2] PSPEG is the viral titre of the positive control analysed with the PEG_{8000} protocol.

Table 1 also shows the results for the analysis of the positive control which was a viral solution without berries. Surprisingly, in this case the results are reversed: the protocol with PEG_{8000} obtained a recovery of 2.769 which is 59.319% of the starting titre, whereas while applying the protocol with proteinase K the viral titre recovered was 0.982 which only accounts for 21.037% of the initial titre.

Tests with human norovirus

Data coming from tests with human noroviruses are encouraging though not conclusive. Starting from a concentration of 176.5 copies per µl it was possible to recover only a small amount of viruses. Observing data reported in Table 2 it was possible to recover a higher number of copies with proteinase K protocol than with the other method but the difference wasn't as clear as for tests with FCV. Again there was an analysis of the concentration of particles remaining in the solution and recovered before the homogenisation of the samples, and the results showed that a mean of 6.623 copies/µl were obtained, i.e. virtually all the viruses remained in the beaker. Given a mean value of 22.73 copies/µl using the proteinase K and 4.89 for PEG_{8000} it is clear that the recovery rates are lower than those obtained with FCV.

Nevertheless, it is important to remember that only 10-100 virions are needed to cause the disease and a concentration of 10^2 copies/µl is very high. The method with proteinase K worked well even with NoV and it was possible to detect low concentrations of viruses but a statistical analysis of data showed that there is no difference between the two methods (significance value 0.06). However, the proteinase K protocol is still preferred because it gives an outcome in less than a day while at least three days are required for the other protocol.

4. Conclusions

Food safety from a microbiological point of view has always been a crucial element in food preparation and processing. However, European legislation doesn't take into account the contamination of food caused by viruses but rather only regulates the presence of microorganisms such as bacteria and moulds. This fact is linked to the lack of standardised methods for the detection of human viruses which can contaminate foodstuffs. Clearly the critical point in every protocol is the virus elution from the surface of foodstuffs and the subsequent purification of the samples from all the possible inhibitors of the most common techniques of detection such as PCR and cell cultures. In this study two different protocols were used to detect viral particles coming from an artificially contaminated foodstuff. One method uses proteinase K to detach the viruses bound to the peel of a mix of berries, whereas the other uses glycine buffer to remove the particles and passages with PEG_{8000} to clean the sample. The fact that the first protocol is fast because it is able to give an outcome in nearly 6 h instead of three days is a point in its favour. The difficulty encountered in the elution of viruses by the food matrix chosen, often reported in literature (Summa *et al.,* 2012), leads to the certainty that the protocol with proteinase K could become a standard method for the elution of viruses from foodstuffs. The results obtained in tests with FCV demonstrate that the protocol with proteinase K has a higher recovery than the other one and provides three times more recovery in percentage on average (23.989% vs. 68.297%). The results are reversed when the data regarding the analysis of the positive control are taken into consideration: in this case it is the protocol with PEG_{8000} which was able to obtain a higher recovery. A possible interpretation of these data is that the protocol which involves PEG_{8000} works better with samples less complex than the berries matrix like lettuce or contaminated water because the matrix has a low interference with the phases of analysis. In contrast, proteinase K without the food is able to act directly on the viral particles damaging the capsid proteins which are not able to infect cells anymore. It is not the purpose of this work to evaluate the capacity of the two protocols in the recovery of viral particles from water or liquid matrices; nevertheless it is important to bear this result in mind because it underlines the fact that for the different matrices which can be contaminated by viruses, different methods would probably be required to fit the matrix and viral characteristics.

Tests conducted with NoV are few and far between, yet they show that even if there isn't a clear statistical difference between the two protocols, the one involving proteinase K is better because it is faster and also recovered a larger amount of particles compared to the other. The concentration registered by real-time PCR is very low and near the detection limit of the apparatus. In the future further tests will be carried out to improve the protocols because at the moment it seems that with the recovery rates calculated the minimum viral concentration required is 10^2 copies/µl to obtain adetection. The improvement of the recovery rate percentages is crucial because in the setting of outbreaks arising from a food contamination, it is very difficult to trace the onset back to a specific food. The problem lies in the small amount of food remaining after the

Table 2. Results of the recovery of viral particles with the two methods. Data are expressed as copies per µl

Protocol	Minimum	Maximum	Mean	Standard deviation
Proteinase K	4.43	60.20	22.73	20.4
PEG_{8000}	1.19	8.67	4.89	2.75

meal. Nevertheless, when searching for the contaminated food it is very important to allow, for example, a batch of frozen raspberries to be recalled from the market as happened in Denmark and France in 2005 (Cotterelle, 2005; Korsager, 2005).

The problem of the contamination of food during its processing or distribution is still relevant and viruses such as NoV are the cause of many outbreaks all over the world. Thus there is an urgent need for an official method for the elution and detection of these viruses. The results obtained demonstrate that the protocol with proteinase K provides good results in a short time coupled both with real-time PCR and cell cultures.

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