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Recovery tests of cytopathogenic viruses from artificially contaminated food samples

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Abstract

The artificial contamination tests were carried out by using a Coxsackie B5 virus of known titration to contaminate vegetable food products (lettuce and berries). The experimental protocol was divided basically into two phases: elution with alkaline pH buffer solution and following concentration of viral particles recovered by using PEG8000 (polyethylene glycol). A third phase of purification with chloroform was introduced between these two steps in order to assess its effect on the yield of the final recovery, and tests were performed in parallel with both the protocols to compare them in terms of recovery efficiency. Elution phase proved to be the most critical, since the viral recovery from food samples during this phase resulted moderate (2.95% and 2.16% respectively in tests without and with chloroform purification phase), data already observed in previous studies. The final concentration phase with PEG8000 recorded average recoveries equalling 0.29% in tests without chloroform and equalling 3.97% in tests with purification phase, thus showing a significant improvement with a lesser interference by the organic material.

Introduction

Acute gastroenteritis is an infection characterized by diarrhoea as its most common clinical manifestation. It may be caused by several protozoal, bacterial and viral agents.

Studies carried out during the 1980s showed that 60% of acute enteritides are of viral origin (Frankhauser *et al.*, 2002): this fact may be explained by the low infecting doses of viruses (10–100 viral particles) compared with the bacterial ones, and by how easily foodstuffs may be contaminated.

The study of the available epidemic data showed that enteric viruses are responsible for 67% of gastroenteric illness episodes among the viruses causing food-borne infections.

The viruses causing gastroenteritis include: Norovirus, Rotavirus, Adenovirus type 40 or 41, Sapovirus and Astrovirus.

Viral gastroenteritis may be easily confused with bacterial gastroenteritis, since they share many of the same symptoms.

Viral gastroenteritis is usually a mild illness, even though it may lead to severe dehydration and other dangerous complications resulting from a combination of severe diarrhoea, vomiting and not drinking enough fluids.

Transmission of enteric viruses typically occurs through the faecal–oral route, but can also occur through person-to-person contact or through ingestion of contaminated food and water.

Foodstuffs involved in the transmission of viral infections to man are many, ranging from water (water may be the cause not only from simply drinking it but also using it to wash other foods) – which represents one of the main infection sources – to milk, meat and fruits, while vegetable products (salad) and fish products (chiefly shellfish) play a particularly important role (Atmar *et al.*, 1995).

In order to link these illnesses to food-borne causes, it is necessary to group the food that may be a carrier of those viruses responsible for the onset of epidemics into proper categories. Although this idea may seem simple, the search of a single classification scheme of foodstuffs represents instead a critical problem.

Generally speaking, a list of the main food products may include different categories: poultry, eggs, pork, beef, milk-dairy produce, fish, shellfish, crustaceans, game, vegetable produce (such as lettuce and maize) and fruits (such as apples, oranges and soft fruits).

Experimental tests carried out with Poliovirus and HAV showed that salad irrigated with contaminated water can adsorb a considerable quantity of viruses on surface, which remains at high levels for several days at 4 °C, and it is not considerably reduced following the washing at home (Durkop, 1992).

Although viruses are acknowledged by now as agents of illnesses acquired through ingestion of contaminated food, it is necessary to identify a lack of methods to isolate them from food matrixes. Their identification in these matrixes or in contaminated waters, in fact, poses an important problem.

Microbiological monitoring is an important means to guarantee food safety. Quick, simple and sensitive techniques for detecting viruses in foodstuffs and water may prove helpful to define the infection causes and source, providing also important information which enables to understand epidemic characteristics (Bouchriti & Goyal, 1992) from the epidemiological point of view. There are effective techniques for the identification in biological samples coming from infected subjects, but this is possible, since the viral loads in these samples are very high, while the loads in foodstuffs are usually low.

The difficulty in defining these techniques depends on several factors: the small-sized virus, the high degree of dilution they undergo in the environment, the virus' ability to form aggregates, the great variation of viruses involved with consequent genic variation, the presence of multiple contaminations with any interfering effects, the food variability and the presence of inhibiting substances.

The ideal method aims at obtaining a final product to undergo molecular biology techniques to identify viruses, which does not interfere with them. Moreover, it will be important to find a method enabling to concentrate the virus from any food sample.

Major breakthroughs were made in the development of techniques envisaging two essential steps: 'sample treatment' from which viruses are extracted and concentrated; and real 'viral identification' carried out through molecular biology techniques or the use of cell cultures.

As regards the viral extraction – phase called *elution* – it is performed by means of a buffer solution. This essential passage is based on the fact that the viral adsorption into tissues or other surfaces is regulated by pH, and this important factor enables to separate the virus during the elution phase, working with a basic pH and providing bonding sites, which compete with those where the virus is adsorbed. Dubois et al. proposed a protocol that includes washing the fruit or vegetable surface with a basic buffer supplemented with a salt, an amino acid and protein (3% beef extract). The proposed washing fluid breaks the electrostatic and hydrophobic interaction between fruit or vegetable surfaces and viruses (Dubois *et al*., 2006).

Later on a clarification phase is reached, which is obtained through centrifugation in order to separate the solid particles (Katzenelson *et al*., 1976; Bouchriti & Goyal, 1992; Bresee *et al*., 2002). Pectinase can be added after clarification to prevent jelly formation during neutralizing of the eluate (Rzezutka *et al*., 2005, 2006; Dubois *et al*., 2006).

At this point the eluted viral substance obtained in this manner must undergo a concentration phase that may be performed by using acid precipitation, filtration flocculation, adsorption with alkaline elution and ultrafiltration, adsorption with alkaline elution and precipitation, elution-precipitation.

However, different techniques combining concentration, purification and genome viral extraction methods have been evaluated (Le Guyader *et al*., 2004; Butot *et al*., 2007; Kim *et al*., 2008; Park *et al*., 2008).

Research purpose

The first goal was to quantitatively assess the recovery of viral particles artificially added to a food product by applying a new elution and concentration protocol, using a cytopathogenic virus that can be easily titrated in cell cultures.

Materials and methods

Tested virus and used cell cultures

Tests of artificial contamination were carried out with Coxsackie B5 virus, strain deriving from a germinal cell isolated from a clinical case.

The viral suspension was prepared by inoculating the culture with 0.1 ml of virus in a 75 cm^2 flask of monkey renal cells deprived of the growth medium and washed with a phosphate-buffered saline washing solution. The flask was incubated in thermostat for 1 hour, at the end of which maintenance medium was added, then placed again into the thermostat at 37 °C until obtaining the maximum cytopathic effect, detectable after 6 days. Later it was frozen at − 20 °C and then defrosted three times. To separate the

supernatant containing the virus from the cells, the culture medium was centrifuged at 2000 rpm for 2 minutes. The viral suspension was purified through ultrafiltration in refrigerated centrifuge at 4 °C at a speed of 3000 rpm on Millipore filters with a 10 000 Da molecular cut. In the end, the virus, held by the filtering membrane, was suspended again in sterile physiological solution and underwent titration by a micromethod calculated in $TCID_{50}$, later stored in freezer at − 80 °C.

Artificial contamination of foodstuffs and procedure of viral recovery

Two hundred millilitre of physiological solution was added with 0.5 ml of viral suspension of known titre, and an aliquot was drawn to determine the initial viral titre.

To contaminate food, 50 g of strawberries or soft fruits were immersed in the physiological solution previously prepared and left for 1 hour.

After this period of time, food was removed aseptically from the physiological solution and a new virus titration was performed in the solution for an assessment on the difference of how much virus was adsorbed in the food.

Later food was treated with a pH 9.2 eluting solution, whose composition (referred to 500 ml) is the following:

- Tris-HCl 100 ml (Cf = 100 mM)
- Glycine 1.88 g (Cf = 50 mM)
- Beef Extract 15 g (3%, 3 g/100 ml)
- $MgCl_2$ 2.35 g (Cf = 50 mM)
- Pectinase 540 µl (Cf = 180 U)

Food was left in contact under agitation for 20 minutes in a 50 ml of this solution. The homogenized material obtained in this manner was centrifuged at 10 000 rpm for 15 minutes, and the supernatant – placed in a flask – was brought to a 7.2 pH with HCl or NaOH. An aliquot was drawn, added with a mixture of antibiotics and eventually titrated for the assessment of the viral recovery during the elution phase.

The remaining part was divided into two aliquots: one was subjected to the virus purification through treatment with chloroform (1/1), performed inside separating funnels and leaving the phases to stratify for about 10 minutes. PEG8000 (polyethylene glycol) in ratio 1:4 was later added, and this was left in contact over night at 4 °C. The other one did not undergo the purification phase, but was directly put into contact with PEG8000.

The following day both the samples were centrifuged at 10 000 rpm for 60 minutes at 4 °C, and the pellet was suspended again in 2 ml of sterile water until its complete dissolution. In the end, after a further centrifugation at 10 000 rpm for 15 minutes at 4 °C, the aqueous phase was recovered to be subjected to titration.

Results and discussion

In the different tests of artificial contamination performed with Coxsackie B5 virus (four without purification with chloroform and eight with purification with chloroform), the recovery percentages of the eluted virus were calculated in terms of the adsorbed virus and the ones of the concentrated virus in terms of the eluted virus.

From the results it is pointed out how the two techniques – both the elution and the concentration – have recovery percentages very low in the first four tests, whilst values improve in the following tests, especially during the concentration phase, where the Chloroform purification was introduced. Recovery percentages of diluted virus in connection with the adsorbed one result from the performed tests ranging between 1.64% and 4.10% (Table 1). An improvement in tests where Chloroform purification was introduced was noticed, where the recovery percentages even reach 8.14% (Table 2), without stressing differences statistically significant, though. As regards the concentration phase with PEG8000 (polyethylene glycol), the recovery percentages of the concentrated virus varied in the two types of tests, showing values that ranged between 0.01%

Table 1 Recovery percentages obtained with Coxsackie B5 before introducing the pre-treatment with chloroform

Test	Eluted (%)	Concentrated (%)
1	4	0.49
2	2.08	0.04
3	1.64	0.64
4	4.10	0.01

Table 2 Recovery percentages obtained with Coxsackie B5 by introducing the chloroform pre-treatment phase in the procedure

Test	Eluted (%)	Concentrated (%)
5	1.07	ND
6	5.89	4.76
7	8.14	3.66
8	0.47	0.52
9	0.04	5.87
10	0.09	3.10
11	1.31	6.31
12	0.3	3.66

(Table 1) and 0.64% and between 0.52% and 6.31% in tests where the purification phase with chloroform (Table 2) was introduced, showing a statistically significant difference ($P < 0.05$).

Conclusions

The results of the performed artificial contamination tests showed first of all that the critical phase is the elution one, which is the detachment of the viral particles adsorbed by the product. In fact, in the two types of performed tests – with and without chloroform purification – the average recovery percentages respectively of 2.95 (1.27) without chloroform and 2.16 (3.086) with chloroform resulted fairly low, however in line with studies conducted earlier, where analyses carried out on fresh strawberries contaminated with hepatitis A virus, Norovirus and rotavirus reached recovery efficiencies ranging between 0.93 and 2.29%.

The final concentration phase with PEG recorded average recoveries between 0.29% (0.31) in tests without chloroform and 3.97% (1.94) in tests with purification phase, showing a considerable improvement with a lesser interference by the organic material.

The work will continue with another important point to estimate the stability of viruses varying the time of exposure and also by applying this protocol to non-cytopathogenic viruses as well, which must include biomolecular techniques such as polymerase chain reaction and real-time polymerase chain reaction.

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