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Evaluation of viral extraction methods on a broad range of Ready-To-Eat foods with conventional and real-time RT-PCR for Norovirus GII detection

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Abstract

Noroviruses (NoV) are a common cause of foodborne outbreaks. In spite of that, no standard viral detection method is available for food products. Therefore, three viral elution–concentration methods and one direct RNA isolation method were evaluated on a broad range of Ready-To-Eat (RTE) food products (mixed lettuce, fruit salad, raspberries and two RTE dishes) artificially seeded with a diluted stool sample contaminated with NoV genogroup II. These seeding experiments revealed two categories of RTE products, fruits and vegetables grouped together and RTE dishes (penne and tagliatelle salads) which are rich in proteins and fat formed another category. The RNA extracts were amplified and detected with two conventional RT-PCR systems (Booster and Semi-nested GII) and one real-time RT-PCR (Real-time GII) assay. A fast direct RNA isolation method detected 10^2 RT-PCRU on 10 g penne and tagliatelle salads with the conventional RT-PCR assays. However real-time RT-PCR was less sensitive for penne salad. A viral elution–concentration method, including a buffer solution for the elution step and one polyethylene glycol (PEG) precipitation step, was able to detect 10^2 RT-PCRU on 50 g frozen raspberries with conventional and real-time RT-PCR assays. Moreover the latter extraction method used no environmental hazardous chemical reagents and was easy to perform.

Keywords: Noroviruses; Ready-To-Eat food; Raspberries; RT-PCR; Real-time RT-PCR

1. Introduction

It is stated that noroviruses (NoV) are the leading cause of non-bacterial gastroenteritis outbreaks of which the foodborne origin is an important source (Mead et al., 1999; Lopman et al., 2003; Prato et al., 2004). Fresh produce is reported to be a major vehicle in foodborne outbreaks (Seymour and Appleton, 2001). Raw and minimally processed produce and mixed salads are part of the Ready-To-Eat (RTE) division. The global trade and the many critical manipulation steps associated with harvesting and processing may account for the frequent implication of these types of RTE foods in outbreaks. The origin of contamination can occur at several points within the farm to fork chain. Fecal contaminated irrigation water used on the fields and infected or asymptomatic food pickers not respecting good hygienic regulations are the main sources of the pre-processing contamination. At the post-harvesting stage, food handlers manually handling these types of food could act as a similar transmission vector as food pickers. Besides fruits and vegetables, RTE dishes, such as caterer meals, are considered as a possible source of viral contamination due to food handlers (Anderson et al., 2001). Deli sandwiches (Daniels et al., 2000), vegetable salads, lunch boxes (Kobayashi et al., 2004; Sakon et al., 2005), bakery products (Kuritsky et al., 1984) are pointed out as causative agents of NoV outbreaks.

NoV infect humans and cause a mild disease with symptoms such as diarrhea, vomiting and nausea which appear within 1 to 3 days after exposure. Nevertheless the frequency of outbreaks and the low infectious dose (100 virus particles or less) (Kapikian et al., 1996) accompanied by the lack of a cell culture (Duizer et al., 2004) reveals the significance to search sensitive extraction and molecular detection methods towards NoV. In order to remove inhibitors of the food hampering the detection, a virus extraction method needs to precede the molecular RT-PCR detection assay. Viral elution–concentration methods separate first the virus particles from the food matrix and include a concentration step by polyethylene glycol (PEG).

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Various reported procedures (Atmar et al., 1996; Jaykus et al., 1996; Traore et al., 1998; Dubois et al., 2002; Le Guyader et al., 2004a) are based on this approach. An alternative viral extraction method is the direct extraction of the total amount of RNA from the food. The comparison of the published methods is difficult due to the different approach of each study.

In this study three elution–concentration methods (Method 1, 2 & 3) and one direct RNA isolation method (Method 4) were compared on a broad range of RTE food matrices spiked with a 100-fold diluted stool sample contaminated with NoV (GII/4) corresponding with around 10^4 RT-PCRU. The four extraction methods were tested in parallel and evaluated with the same amplification/detection assays. The extraction methods, detecting the high NoV GII/4 inoculum, were further evaluated with lower inoculation levels (10^3 to 10 RT-PCRU). Method 1 and Method 2, based on respectively Le Guyader et al. (2004a) and Dubois et al. (2002), were interesting to compare because of the differences in the type of washing solution to elute the virus particles from the food matrix and the order in which purification and precipitation steps were performed. Method 3 is a combination of washing (Traore et al., 1998)/concentration (Dix and Jaykus, 1998; Leggitt and Jaykus, 2000)/purification (Atmar et al., 1995) steps reported in earlier studies which showed to be efficient. The viral RNA obtained with Method 3 was released by proteinase K which attacks the capsid proteins of inactivated virus particles (Nuanualsuwan and Cliver, 2002). A cationic detergent, CTAB was incorporated because of the ability to remove inhibiting components interfering with the RT-PCR assay (Jiang et al., 1992).

The direct RNA isolation method (Method 4) used Trizol and is comparable with a method reported by Schwab et al. (2000). Two conventional RT-PCR reactions were included to evaluate the extraction methods and to have a duplicate confirmation of each result. In addition real-time RT-PCR was used to test its applicability on RTE foods.

Method 1, 2 and 4 were not exactly performed as they were reported in literature. Modifications were applied in order to make the extraction methods easier and straightforward in order to establish simple viral extraction methods which could serve as a routine screening method for RTE foods.

2. Materials and methods

2.1. Artificial contamination of RTE foods

The artificial contamination of the RTE foods was done with a stool sample originating from an outbreak which was confirmed to be contaminated with GII/4. The stool sample was kindly provided by the National Institute for Public Health and the Environment (RIVM) — the Netherlands. The 10% stool sample suspension was subjected to centrifugation (855 ×*g*, 20 min, room temperature (RT)). The supernatant was transferred to a new tube. From this solution tenfold dilutions were made in PBS (145 mM NaCl, 7.7 mM Na_2HPO_4, 2.3 mM NaH_2PO_4, pH 7.4). The samples were stored at 2 °C. The viral RNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer from 100 µl of each tenfold dilution of the stool sample. Endpoint dilution RT-PCR determined that 10^7 RT-PCRU (units)/ml of NV GII/4 were present in the original stool sample.

Fruit salad, mixed lettuce and two complete RTE dishes: (1) vegetarian penne with pesto and (2) tagliatelle with chicken and vegetables were retrieved from a local supermarket. Frozen raspberries were kindly provided by a local food manufacturer. These samples were artificially contaminated with 50 µl of the 100-fold dilution of the stool sample (corresponding with around 10^4 RT-PCRU). The extraction methods included a non-inoculated food sample of each type of food matrix that served as a negative control.

Further evaluation of the extraction methods enabling the detection of the high inoculum was performed on frozen raspberries, penne and tagliatelle salads seeded with 100 µl of tenfold serial dilutions of the same stool sample containing GII/4 (corresponding with 10^3 to 10 RT-PCRU/100 µl). All extraction methods were performed in duplicate.

2.2. Viral extraction methods

An overview of the viral extraction methods compared in this study is shown in Fig. 1. Methods 1, 2 and 3 are viral elution–concentration methods. The virus particles are firstly eluted from the food matrix and then precipitated using PEG. Method 4 directly extracts the total amount of RNA from the food matrix. Variants of Method 2, 3 and 4 were included. Details of the extraction methods are described underneath.

2.2.1. Method 1

This method was based on a previously reported method by Le Guyader et al. (2004a). 50 g of food product was washed for 20 min with 30 ml 0.05 M glycine (Acros organics, Geel, Belgium)–0.3 M NaCl (Sigma, Steinheim, Switzerland), pH 9.5 (adjusted with 10 M NaOH (Sigma)) in a stomacher bag with filter compartment. The filtrate was kept. Further, 22 ml of chloroform/butanol (1:1 vol/vol, VWR international, Fontenay sous Bois, France/Sigma) was added to the filtrate. After centrifugation (10,000 ×*g*, 20 min, 4 °C), the aqueous phase was separated. Twenty ml of PEG 6000 (24% wt/vol, Fluka Chemie, Buchs, Germany) — 1.2 M NaCl was added. The samples were placed on a shaking platform during one hour. By centrifugation (10,000 ×*g*, 30 min, 4 °C) the virus particles were precipitated. After digestion with 3 ml proteinase K for 30 min at 56 °C (0.2 mg/ml in 50 mM Tris–HCl pH 8; 1 mM $CaCl_2$, Sigma), 3 ml phenol/chloroform/isoamylalcohol (25:24:1, Sigma) was added. The samples were centrifuged (10,000 ×*g*, 20 min, 4 °C) and the aqueous phase was transferred to microcentrifuge tubes. Finally, ethanol (VWR international, Leuven, Belgium) was added to precipitate the RNA from the aqueous phase by centrifugation (9503 ×*g*, 20 min, RT). The pellet was dissolved in 100 µl of RNase, DNase free water and then further purified with the RNeasy Mini kit (similar as was done for the RNA isolations from the serial diluted stool samples).

2.2.2. Method 2

This method was based on a previously reported method by Dubois et al. (2002). Briefly, 50 g of food product was washed