Comparison of automated extraction methods for the detection of hepatitis A virus and norovirus in mussels

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Summary
Noroviruses and hepatitis A virus are the pathogens most frequently involved in non-bacterial gastroenteritis and hepatitis worldwide. They are mainly transmitted via the faecal–oral route, direct person-to-person contact or through the consumption of contaminated water and foods. In food virology, detection methods of these viruses are currently based on real-time reverse transcription-polymerase chain reaction (RT-PCR). A crucial step in this process is the acid nucleic extraction, since its performance can negatively influence viral detection and thus give false negative results. The aim of this study is to evaluate the efficiency of 2 automated extraction systems, MagMAX Express and EZ1 Advanced XL, in recovering hepatitis A virus and norovirus RNA from mussels. In the present study, we used mengovirus as a process control to assess the efficiency of the extraction process. Samples were tested for mengovirus, hepatitis A, and norovirus by real time one-step RT-PCR assay. Our data indicates the MagMax Express is the better system to extract hepatitis A and norovirus RNA from mussels since its extraction efficiency was higher ($p < 0.05$) than EZ1 Advanced XL.

Confronto tra metodi di estrazione automatizzata per l'individuazione del virus dell'epatite A e del norovirus nelle cozze

Parole chiave
Bivalvi,
Epatite A,
Mengovirus,
Cozze,
Norovirus,
Real-time RT-PCR.

Riassunto
Il norovirus e l'epatite A sono i virus che più frequentemente causano in tutto il mondo le gastroenteriti non batteriche e le epatiti. Si trasmettono principalmente attraverso la via oro-fecale, il contatto diretto tra persone o consumando acqua e alimenti contaminati. Per rilevare la presenza di questi virus si utilizza una real-time reverse transcription-polymerase chain reaction (RT-PCR). In questo processo, l'estrazione dell'acido nucleico è un passaggio cruciale perché può influenzare negativamente la rilevazione virale, dando false negatività. Lo scopo di questo studio è valutare l'efficienza di 2 sistemi di estrazione automatizzata, MagMAX Express ed EZ1 Advanced XL, per rilevare nelle cozze la presenza dell'RNA del virus dell'epatite A e del norovirus. I campioni sono stati testati con real time one-step RT-PCR per epatite A, norovirus e mengovirus, utilizzando quest'ultimo come controllo per valutare l'efficienza del processo di estrazione. I dati hanno dimostrato che il MagMax Express è il sistema migliore, poiché raggiunge un'efficienza di estrazione più elevata ($p < 0,05$) rispetto a EZ1 Advanced XL.

Introduction

Viral food-borne outbreaks are an increasingly important public health concern worldwide, incurring a considerable economic burden.

Bivalves are filter-feeder organisms that can concentrate and retain viruses from the surrounding polluted waters, thus becoming a dangerous food with the potential to compromise human health (Lees 2000, Shieh *et al.* 2000).

In compliance with the current EU standard, the sanitary quality of mussels is entirely based on evaluating bacterial indicators of faecal contamination (Reg. 2073/2005 and Reg. 1441/2007)[1], despite a body of literature that indicates that bacteria are not reliable indicators of the presence of viruses in shellfish (Croci *et al.* 2000, Lees 2000, Fusco *et al.* 2013).

Norovirus genogroup I (NoV GI), genogroup II (NoV GII) and hepatitis A virus (HAV) are the most common causes of acute, non-bacterial gastroenteritis and hepatitis worldwide. These viruses can be readily transmitted via the faecal-oral route, by person-to-person contact or via contaminated water, food, and from environment (Koopmans *et al.* 2004). Numerous viral outbreaks involving noroviruses (NoVs) and HAV have been associated with the consumption of raw or undercooked bivalve shellfish harvested in faecal polluted waters (Le Guyader *et al.* 1996, Lees 2000, Shieh *et al.* 2000).

To date, NoVs cannot be propagated efficiently using conventional cell culture methods while HAV grows poorly, thus detection of both NoVs and HAV in mussels relies on molecular biological methods, such as reverse transcription-polymerase chain reaction (RT-PCR) (Le Guyader *et al.* 2000, Comelli *et al.* 2008). The detection of enteric viruses in food is difficult due to the low level of viral contamination as well as the presence of substances that can inhibit PCR amplification (Lee *et al.* 2012, Maunula *et al.* 2013, Suffredini *et al.* 2014).

A primary step in the RT-PCR is the nucleic acid extraction, since its performance can affect viral detection, giving false negative results, especially if the target virus is present at low concentrations. For this purpose, process control viruses are added to the sample just before virus extraction in order to monitor the efficiency of the entire extraction procedure (Hennechart-Collette *et al.* 2015).

In our study, we evaluated and compared the performance of 2 RNA/DNA extraction automated systems, MagMAX Express (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.), and EZ1 Advanced XL (Qiagen, Hilden, Germany), using recombinant mengovirus (vMC_0) as process control. Results allowed us to establish which system is more sensitive and efficient in recovering HAV and NoVs from mussels. The chosen extraction system method will be used in further experiments on the research of viral contaminants in bivalves.

Materials and methods

Sample collection and positive controls

We analysed a total of 30 samples of mussels (*Mytilus galloprovincialis*) collected in the province of Naples, Southern Italy, by the local public health authority (ASL) from June 2014 to April 2015. Mussels were collected from harvesting plants for surveillance routine analyses. The National Reference Laboratory for Viral Contamination in Bivalve Molluscs, Istituto Superiore di Sanità, Rome provided HAV, NoV GI, NoV GII and vMC_0, which were used as positive controls.

Virus extraction

All samples were analysed according to the UNI CEN ISO/TS 15216-2:2013[2].

For each sample, 2 g of hepatopancreas (approximately 10 mussels) were collected, chopped finely, and combined with 2 ml of Proteinase K solution (0.1 mg/ml) (Qiagen Hilden, Germany). The mixture was then spiked with 10 µl of vMC_0 process control ($1.6x10^2TCID_{50}$/ml). Samples were first vortexed and then incubated at 37 °C under shaking (320 rpm) for 1 hour in order to dissolve tissues. Samples were then incubated at 60 °C for 15 minutes before being centrifuged at 3000 g for 5 minutes. The supernatant was subsequently collected and adjusted to a final volume of 3 ml with sterile Phosphate-Buffer Saline.

Viral RNA extraction

Two automated commercial systems, MagMAX Express (MM) and EZ1 Advanced XL (EZ1), were used to performed RNA extraction. Manufacturer's

[1] Regulation (EC) N. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. *OJ*, **L 338**, 22.12.2005, 1-26.
Regulation (EC) N. 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs. *OJ*, **L 322**, 7.12.2007, 12-29.

[2] UNI CEN ISO/TS 15216-2:2013. Microbiology of food and animal feed - Horizontal method for determination of Hepatitis A virus and Norovirus in food using real-time RT-PCR.

instructions for nucleic acid extraction of virus were carefully followed. In the MM system, 300 µl of treated hepatopancreas homogenates were processed and the sample was eluted in 90 µl elution buffer. In the EZ1 system, extraction was performed on 400 µl of viral extract and nucleic acids were eluted in 60 µl elution buffer. Both systems are based on magnetic-particle technology and involve the following steps: the lyses of the sample; the binding of DNA/RNA to a coated magnetic resin in the presence of chaotropic salts; the washing of the resin to remove undesirable compounds; and the elution of RNA from the particles in a low-salt solution. All eluted samples were added with 1U/µl RNase inhibitor (Promega Corporation, Madison, WN, USA), and stored at - 80 °C until use.

Detection of viruses by real-time RT-PCR

Detection of HAV, NoV GI, NoV GII and vMC_0 was carried out by qualitative 1 step real-time RT-PCR according to the UNI CEN ISO/TS 15216-2:2013.

Reactions were performed on the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in 25 µl reaction mixture containing 5X RNA UltraSense™ master mix, 20X of RNAUltraSense™ enzyme mix, (RNA UltraSense™ One-Step Quantitative RT-PCR System, Thermo Fisher Scientific, Waltham, Massachusetts, USA), 0.5 pmol/µl forward primer, 0.9 pmol/µl reverse primer, 0.25 pmol/µl probe (Table I) and 5 µl of extracted sample. All primers and probes were purchased from IDT (Coralville, Iowa, USA).

Each reaction included a positive control, a negative control containing all the reagents except the RNA template, and an external control RNA to evaluate the presence of PCR inhibitors (Le Guyader *et al.* 2009, Suffredini *et al.* 2014). Each sample was analysed in triplicate, both undiluted and 10 fold diluted.

The one step RT-PCR thermal profile involved 60 minutes reverse transcription at 55 °C, followed by a 5 minute denaturation step at 95 °C, and 45 cycles of 15 seconds at 95 °C, 1 minute at 60 °C and 1 minute at 65 °C. Samples were considered positive in the presence of a cycle threshold value (Ct) ≤ 43 in at least 2 replicates according to the UNI CEN ISO/TS 15216-2:2013 that suggests a thermal profile with 45 cycles.

Efficiency of the extraction procedure

For each system, the extraction efficiency (R) was calculated comparing the Ct value of mengovirus in sample with the Ct value of mengovirus stock according to the equation $R = 2^{-\Delta Ct}$, applying correction factors that take into account the different volume of the sample loaded and elution volume (Di Pasquale *et al.* 2010, Fleige *et al.* 2006. Suffredini *et al.* 2014). According to the UNI CEN ISO/TS 15216-2:2013, R was considered acceptable when was >1%.

Statistical analysis

Mean values of extraction efficiency obtained with both systems were compared by T-Student Test to determine the statistical significance. Significance was accepted in case of a 2 tailed p-value ≤ 0.05. Statistical analysis was carried out with IBM® SPSS® Statistics 20 software (IBM corp., Armonk, NY, USA).

Real-time RT-PCR performance evaluation

Amplification efficiency (E) of the real-time RT-PCR

Table I. *List of primers and probes used in the real-time RT-PCR.*

Primers and probes	Sequence (5'-3')	Reference
HAV68 (Fw)	TCACCGCCGTTTGCCTAG	Costafreda *et al.* 2006
HAV240 (Rev)	GGAGAGCCCTGGAAGAAAG	
HAV150 (Probe)	FAM-CCTGAACCTGCAGGAATTAA-MGBNFQ	
QNIF4 (Fw)	CGCTGGATGCGNTTCCAT	da Silva *et al.* 2007, Svraka *et al.* 2007
NV1LCR (Rev)	CCTTAGACGCCATCATCATTTAC	
NVGG1p (Probe)	FAM-TGGACAGGAGAYCGCRATCT-TAMRA	
QNIF2 (Fw)	ATGTTCAGRTGGATGAGRTTCTCWGA	Loisy *et al.* 2005, Kageyama *et al.* 2003
COG2R (Rev)	TCGACGCCATCTTCATTCACA	
QNIFs (Probe)	FAM-AGCACGTGGGAGGGCGATCG-TAMRA	
Mengo110 (Fw)	GCGGGTCCTGCCGAAAGT	Pinto *et al.* 2009
Mengo209 (Rev)	GAAGTAACATATAGACAGACGCACAC	
Mengo147 (Probe)	FAM-ATCACATTACTGGCCGAAGC-MGBNFQ	

for mengovirus detection was calculated from the standard curves as previously described (Amoroso *et al.* 2011) using the 2 extraction systems under study. For the scope, real-time RT-PCR was performed on samples of water spiked with 10-fold serial dilutions of vMC_0 (from $1.6x10^4$ to 1.6 $TCID_{50}$/ml), extracted with both MM and EZ1.

Results

Assessment of the two automated systems

EZ1 Advanced extraction ability

Results showed that EZ1 extraction efficiency ranged from 0.16% to 40.81% with 22 out of 30 samples reaching an acceptable (> 1%) R value. As indicated in Table II, mean R of this system was 5.11%.

Table II. *Extraction efficiency of the two automated systems on 30 samples.*

	MAGMAX Express (MM)	EZ1 Advanced XL (EZ1)
Extraction efficiency (mean % + SD)	25.55 + 35.86	5.11 + 9.08
Samples with R < 1%	2 (6.6%)	8 (26.6%)
Samples with 1% < R < 10%	9 (30%)	18 (60%)
Samples with R > 10%	19 (63.3%)	4 (13.3%)

R indicates extraction efficiency: R < 1% poor, 1% < R < 10% acceptable, R > 10% good (Vilarino *et al.* 2009).

MagMax Express extraction ability

MagMax exhibited values of R ranging from 0.77% to 138%. In details 28 out of 30 samples reached an acceptable (>1%) R value. Mean R of this system was 25.55% (Table II).

Real-time RT-PCR amplification efficiency evaluation

We evaluated amplification efficiency of the real-time RT-PCR spiking with mengovirus at different concentrations (from $1.6x10^4$ to 1.6 $TCID_{50}$/ml) both water and mussel samples.

The Analyses of Ct values showed comparable values for MM and EZ1 in water. Whereas, the same experiment conducted by spiking mussels with serial dilution of mengovirus showed higher Ct values (more than 2 log difference); no Ct values for matrix samples spiked at lowest concentrations ($1.6x10^1$ and 1.6 $TCID_{50}$/ml) were detected.

Amplification efficiency in water samples was comparable for both systems (E = 104% for EZ1 and E = 107% for MM); while it resulted lower in spiked matrix, giving nonetheless satisfactory results (E = 85%) (Figure 1).

Detection of HAV and NoVs in field samples

The nucleic acid of the 30 samples extracted by both systems were also investigated for the presence of HAV, NoV GI, and NoV GII according to

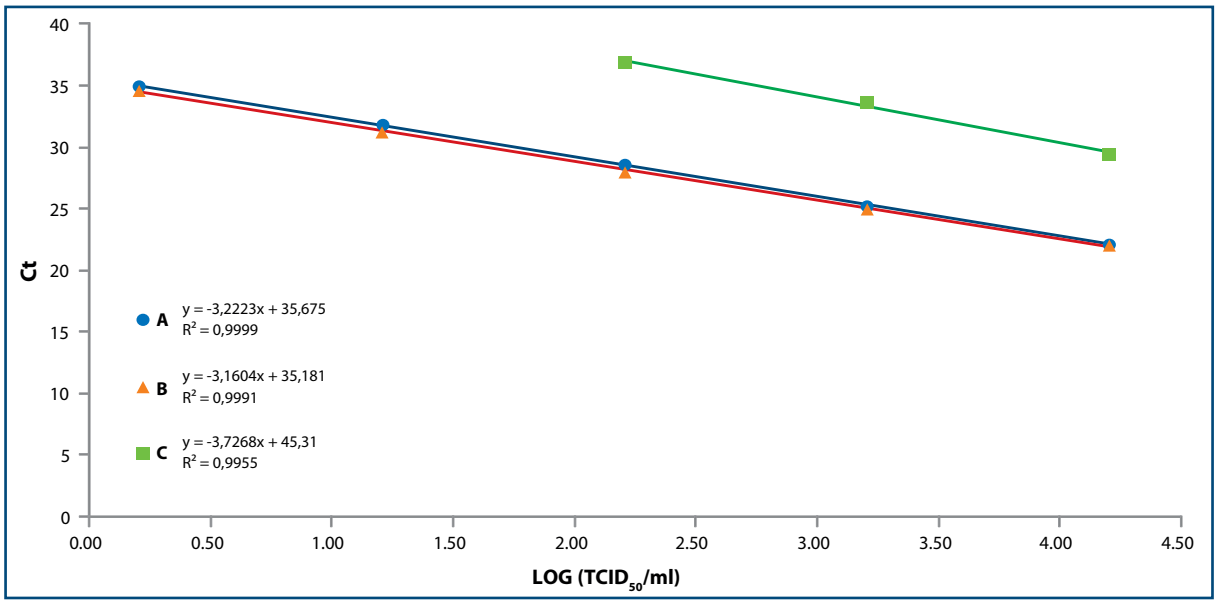

Figure 1. *Standard curves obtained spiking water (curves A and B) and hepatopancreas (curve C) with decreasing concentrations of vMC_0.* Samples were extracted with EZ1 (A) and MM (B and C) and amplified by real-time RT-PCR. A: E = 104%, detection limit 1.6 $TCID_{50}$/ml; B: E = 107%, detection limit 1.6 $TCID_{50}$/ml; C: E = 85%, detection limit $1.6x10^2$ $TCID_{50}$/ml.

Table III. *Real time RT-PCR mussels positivity (Ct values) to HAV, NoV GI and NoV GII (on 30 samples).*

	Sample	HAV Ct	NoV GI Ct	NoV GII Ct
EZ1	A	41	39.4	36
	B	42	42	35,5
	C	neg	42,5	38,5
	D	neg	41	42
	E	neg	neg	37,5
	F	neg	neg	39
	G	neg	neg	35
MagMax	A	40	38	35
	B	40,5	41,5	35
	C	42,5	42	38
	D	43	40,5	41,5
	E	neg	neg	37
	F	neg	neg	38
	G	neg	neg	34

Ct is cycle threshold. Samples C and D were positive to HAV only with MM.

the UNI CEN ISO/TS 15216-2:2013. Results showed that 23 samples (76.6%) were negative for all viral targets with both extraction systems (Table III). Seven samples (22%) were positive for NoV GII. Of these samples, 4 (14%) resulted also positive for NoV GI and HAV, when extracted with MM, while 2 of them were not further confirmed for HAV positivity when extracted with EZ1. The positivity of samples to NoV GI and NoV GII was the same with both extraction systems.

Discussion

Detection of viruses in bivalves is mainly influenced by the low viral concentration and by the presence of RT-PCR inhibitors. Therefore, it is of a primary importance to choose an efficient extraction protocol prior to RT-PCR detection with the aim to reduce the risk of false negative results. Furthermore, analysis of enteric viruses in complex matrices like food should always include a process control monitoring the entire sample treatment (Uhrbrand *et al.* 2010), since its use contributes to assure proper interpretation of results when detecting a food pathogen (Hennechart-Collette *et al.* 2015).

This work describes a comparison of nucleic acid extraction protocols carried out with two automated systems, EZ1 and MM, using vMC_0 strain as a process control, with the aim to compare extraction efficiency and real-time RT-PCR performance in mussels. This control virus has been chosen, in accordance with literature, because it shares structural features with HAV (Costafreda *et al.* 2006, Uhrbrand *et al.* 2010), and since it showed similar behaviour to NoVs in bioaccumulation studies in oysters (Le Guyader *et al.* 2009) and mussels (Uhrbrand *et al.* 2010, Comelli *et al.* 2008).

Mengovirus is the process control virus suggested by the UNI CEN ISO/TS 15216-2:2013 and has been already used successfully when analysing different types of water (Amdiouni *et al.* 2013), shellfish (Suffredini *et al.* 2014), and various food samples: fresh leafy greens, soft red fruits and fresh produce such as tomatoes, cucumbers and fruit salads (Costafreda *et al.* 2006, da Silva *et al.* 2007, Comelli *et al.* 2008, Baert *et al.* 2011, Fuentes *et al.* 2014).

The same extraction efficiency was observed in water samples spiked with mengovirus; while when mengovirus RNA was extracted from mussel spiked hepatopancreas the Ct values were much higher than from spiked water. In fact, it is well known in literature, that mussels and shellfish are rich in sterol, thus the presence of matrix inhibitors could be responsible for suboptimal RNA recovery (Hennechart-Collette *et al.* 2015).

As to the experiments carried out on field samples, their recovery rate (Table II) shows that extraction efficiency of EZ1 was under 1% in 26.6% of samples (8/30). Instead, the MM shows much more satisfying results with 93.3% of samples (28/30), exhibiting an extraction efficiency > 1%.

When comparing mean R, MM value is > 10% (25.55%, Table II) and can, therefore, be considered good in accordance with literature (Vilarino *et al.* 2009); on the contrary EZ1 mean R (5.11%) can be classified as acceptable give that the R value is $1 < R < 10$ (Vilarino *et al.* 2009).

Since, with the use of the external control, we did not observe inhibition during our experiments, the differences in recovery rates observed could be attributed to some diversity in the 2 extraction protocols. Although both systems are based on magnetic-particle technology, they diverge in lysis solutions (Proteinase K in the EZ1 and guanidinium thiocyanate-based solution in the MM) and number of washing steps (3 in the EZ1 and 4 in the MM). The shorten washing process of the EZ1 might be responsible of the incomplete removal of matrix inhibitors, thus resulting in lower extraction efficiency (Aebischer *et al.* 2014).

Furthermore, we observed that 2 field samples resulted positive to HAV only when extracted with MM (Table III). These data confirm that the extraction method is a key factor to detect pathogen viruses, like HAV, which can cause severe disease to the consumer when present even at very low concentrations (Lees 2000).

The 2 systems under study are both automated (although to a different extent). While EZ1 is a close platform that allows for high standardization; but

it can only process up to 14 samples; MM requires some operator steps; but it can process up to 96 samples in a single working session.

The MM was found to be a more efficient method allowing for a more sensitive recovery of viral RNA from mussels. Moreover, as it can to process 96 samples, it can significantly decrease laboratory time. More experiments, with a greater number of samples and the different viral targets will be carried out to confirm our results.

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