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Comparison of three guanidinine thiocyanate RNA extraction methods applied to shellfish extract for enteric virus detection

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Abstract

Human enteric viruses are transmitted through water and food. It's therefore necessary to include the research of these agents in the food and water sanitary quality analysis. Most of these viruses are not culturable. Gene amplification methods such as NASBA and RT-PCR are used for routine analysis. These methods of detection are possible only if there is an adapted procedure for extracting nucleic acids especially RNA. We proposed here to evaluate the quality of the three RNA extraction protocols using the guanidine thiocyanate. Two of the considered extraction methods are based on the work of Chomczynski and Sacchi (method I and method II). A third one which is a modification in order to reduce the working time was also applied (method III). Extraction tests were performed on mussel extract after concentration with PEG / NaCl . The quality of extraction is evaluated by spectrophotometry on the criteria "yield " and " purity" . The results show that method I and method II are equivalent and suitable and the method III was not appropriate for this type of samples.

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Introduction

The human enteric viruses are excreted in the stool of infected persons. They are non-enveloped viruses that are able to persist in the environment and remain infectious for months. Water and contaminated food are the main vectors of these agents since they are transmitted by faecal-oral route (Jiménez-Clavero *et al.*,2006). Bivalves are filter-feeders that bioaccumulate viral agents of their aquatic environment. More, these shellfish are often eaten raw or slightly cooked (Karamoko *et al.*, 2005). Frequent outbreaks of viral gastroenteritis are associated with raw shellfish consumption. The virological quality control of food continues therefore to grow (Le Guyader *et al.* , 2007). The main difficulty is that most of these viruses are not cultirable. Therefore, molecular biology techniques such as PCR and NASBA are widely used (Richard *et al.*; 1999). These techniques cannot be applied correctly when the virus concentration and nucleic acid extraction (RNA particularly) steps are not efficient. In environmental samples the human enteric viruses can exist only sporadically in lower concentration than in the relevant clinical samples (Santos and Gouvea; 1994). Therefore the in-house methods of extraction turn out to be less effective. Sometimes the yield and purity are not sufficient (Bouchriti *et al.*, 1993; Boch, 1998).

We consider three RNA extraction methods. They are all based on the use of guanidine thiocyanate a chaotropic agent used for the RNA extraction purpose firstly by Chomczynski and Sacchi, (1987). Guanidine thiocyanate has also lysis and nuclease inactivating properties. From the original guanidine thiocyanate method derived variants that have the advantage of reducing the RNA isolation steps (Boom *et al.,* 1990). These methods are usually used on tissue samples for extraction of total RNA with success. In food and water virology, concentration steps lead to concentrates that are poor in RNA when compared to tissue samples. To succeed therefore this critical stage, reserachers use mostly commercial RNA extraction kits that are supposed to be more efficient. However the use of commercial RNA Kits increases significantly the cost of the analysis made in environmental virology. Our goal is to make a comparative study of the efficacy of the three considered extraction methods on shellfish samples, mussels in this case treated by the PEG method of concentration (Kingsley *et al.* 2002)

Material and methods

For virus concentration from shellfish (mussels) samples, direct glycine elution was applied according to Kingsley *et al.* (2002), with the difference that the PEG8000 pellet was resuspended in 2 ml 0.15 M Na2HPO4,pH 9 buffer and distributed in 200 µl aliquots stored at -20°C before nucleic acid extraction. RNA was extracted by three isolation methods base on guanidine thiocyanate.

Table 1. Composition of D solution.

Components	Quantity	Final Concentration
Guanidinium Thiocyanate	47.26 g	4.0 M
1.0 M Sodium Citrate	2.5 ml	25 mM
10% Sarcosyl	5.0 ml	0.5%
2-mercaptoethanol	720 µl	0.1 M
DEPC-H_2O	100 mL	QSP

RNA Extraction by the method I : AGTC by Chomczynski et Sacchi, (1987)

This is the original approach of RNA isolation proposed by Chomczynski et Sacchi, (1987). Briefly, to 100µL shellfish extract obtained after the application of glycine /nacl virus concentration method, 500 µl of D solution (table 1.) was added followed sequentially by :60µL of a 2M sodium acetate solution, pH 5.2 and 500 µL of phenol:chloroforme:isoamyl alcool (25:24:1). This was incubated on ice during 15 min then centrifugated at 10000 g during 20 min. the

resulting pellet was transferred in a new sterile 1.5 ml microtube and one volume of isopropanol was added before putting on ice for two hours precipitation at – 20°C. After precipitation, a 30 MIN centrifugation at 12000 g was performed. The pellet was resuspended in 300µL of D solution. To the obtained suspension one volume of isopropanol was added and it was let precipitate at – 20°C during one hour. Centrifugation was done subsequently at 12000 g for 30 min followed by the pellet wash with 600 µL of 75% ethanol and centrifugating at 12000 g during 5min. The final pellet was ressuspended in 30 µL of DEPC treated sterile distilled water.

Table 2. Composition of T Reagent.

components	Quantity	Final Concentration
Satured phenol pH 4.3	38 mL	38%
Guanidium thiocyanate	11.816 g	0.8 M
Ammonium thiocyanate	7.612 g	0.4 M
sodium acetate, pH 5.0	3.34 mL 3M solution	0 .1 M
Glycerol	5.0 mL	5 %
DEPC-H_2O	Enough for	100 mL

RNA extraction by modified AGTC (method II)

The method II is a modification of the AGTC method by his principal author (Chomczynski, 1993). The objective is to reduce the number of steps of the original method by using T reagent (table 2.) a monophasic reagent that contains phenol and the chaotropic agent. This protocol is summarized as follows. 200µL of shellfish extract was put in a 1.5 mL sterile microtube, 800 µL of T reageant, 200µL chloroform:isoamyl alcohol (24:1) was added sequentially. After ten manual inversions it was incubated on ice during 15 min then centrifugated at 10 000 g during 15 min at 4°C. The aqueous supernatant was transferred in a new sterile microtube then, an equal volume of isopropanol was added before 2h precipitation at -20°C. This was centrifugated at 14 000 g for 15 min at 4°C. The pellet was air dried before resuspending in 30 µl of DEPC treated sterile distilled water.

Table 3. Z reagent**.**

components	Quantity	Final concentration
D Solution without 2-mercatoethanol	15 mL	
2-mercatoethanol	10 µL	0.1 M
8-hydroxyquinoline	15 mg	0.1%
sodium Acetate 3M pH 5.2	1.0 mL	0.2 M
Satured phenol pH 4.3	15 mL	

Method III using "Z reagent"

This is our adaptation of a homemade monophasic reagent with guanidine and phenol for quick RNA isolation (Greene, 1993; Weber *et al.*, 1998). In this case 100 µL of shellfish extract was added to 200µL of Z reagent (table 3.), homogenization was done by ten manual inversion. This was followed by the addition of 60 µL of chlroform:isoamyl alcohol (24:1) and vortexing for one min before incubating on ice for 20 min. subsequently centrifugation was done at 14000 g during 15 min. The aqueous supernatant was transferred to a new microtube and equivalent volume of isopropanol was added for 2h precipitation at -20°C. After precipitation, centrifugation at 10000 g during 20 min was performed. The resulting pellet was air dried and resuspended it in 20µL of sterile distilled water treated by DEPC.

RNA extraction quality control

To evaluate the extraction, we used Biorad ® SmartSpec Plus Spectrophotometer to measure

optical density at 260 nm and 280 nm wavelengths. Nucleic acids absorb stalwartly at 260 nm when the protein absorbs strongly at 280 nm. For quantification, Beer-Lambert law A_{260} = e*C*l is used with a quartz cuvette. the optical path(l) of the cuvette is 1 cm, we determined the concentration C as follows: C =A_{260}* 40 µg/ml

RNA extraction with each method was evaluated by measuring the optical density OD260 (at 260 nm) and OD280 (at 280 nm) respectively corresponding to RNA and protein absorption wavelengths. The OD260/OD280 ratio is calculated to discern the RNA contamination by proteins (low Ratio). The optical density OD260 allowed to calculate the amount of RNA collected using the law of Beer-Lambert (de Silva Gesteira *et al.*, 2003) the analysis covered 14 samples for methods I and method II then seven samples for method III.

For the comparison of the three methods of RNA extraction The General Linear Model procedure was used with the SAS software to compare the three methods (SAS Institute, 1992).

Results and discussion

Measured OD and OD260/OD280 ratios

The OD measurements results and OD260/OD280 ratios are reported in Table 4, 5, 6.

The General Linear Model procedure was used with the SAS software to compare methods (SAS Institute, 1992).

For the criterion 1 (RNA yield) the difference between the three methods is highly significant. The comparison of means was made by the LSD test (Least Significant Difference). It shows that the difference between methods I and II was not significant while they are both significantly** better than method III. For the Criterion 2 (purity of the extracted RNA OD260/D280) for this criterion the difference between the three extraction methods is not significant. When we compare the average LSD test showed that only two means are significantly different. ** They are OD260/OD280 means of methods II and III.

Table4. Quality evaluation of RNA extraction by method I.

OD280	OD260	OD260/ OD280	RNA µg /100µL
0.174	0.340	1.95	13.6
0.188	0.261	1.39	10.44
0.135	0.240	1.78	9.6
0.180	0.300	1.67	12
0.185	0.262	1.41	10.48
0.218	0.296	1.35	11.84
0.218	0.298	1.37	11.92
0.174	0.320	1.83	12.8
0.186	0.289	1.55	11.56
0.187	0.288	1.54	11.52
0.175	0.292	1.67	11.68
0.175	0.299	1.71	11.96
0.189	0.290	1.53	11.6
0.192	0.300	1.56	12
Mean		**1.60**	**11.62**

Table5. Quality evaluation of RNA extraction by method II.

A_{280}	A_{260}	A_{260}/ A_{280}	ARN µg /100µL
0.153	0.300	1.96	12
0.168	0.260	1.55	10.4
0.174	0.328	1.89	13.12
0.181	0.269	1.49	10.76
0.173	0.282	1.63	11.28
0.165	0.293	1.78	11.72
0.185	0.317	1.71	12.68
0.189	0.323	1.71	12.92
0.172	0.308	1.79	12.32
0.185	0.242	1.31	9.68
0.180	0.300	1.67	12
0.185	0.262	1.42	10.48
0.194	0.298	1.54	11.92
0.203	0.300	1.48	12
Mean		**1.63**	**11.67**

Choice of protocols

These techniques were selected from the ones that can be fully conceived in the laboratory without the use of commercial kits (Bianchi *et al.*, 2011). These

kits usually represent a significant part of the cost of virology water and food. The three studied techniques are usually used on rich RNA samples (tissue and cell culture) (Arnal *et al.* ,1999; Atmar *et al.*, 1995; Boch, 2001). These methods all derived from the work of Chomczynski and Sacchi (1987). They are compared with respect to both criteria and ratio OD280/OD260 amount of RNA collected.

Table6. Quality evaluation of RNA extraction by method III.

	OD_{280}	OD_{260}	OD_{260}/OD_{280}	RNA µg/100 µL
	0.133	0.183	1.37	7.32
	0.142	0.198	1.40	7.92
	0.127	0.208	1.64	8.32
	0.151	0.197	1.30	7.88
	0.134	0.202	1.51	8.08
	0.136	0.193	1.42	7.72
	0.145	0.217	1.5	8.68
Mean			**1.45**	**7.99**

OD280/OD260 ratio (criterion1)

Methods I (AGTC) and II (with reagent T) gave satisfactory results with OD280/OD260 ratio were 1.6 and 1.63 respectively. The amount of RNA extracted from the concentration after treatment of mussel samples is on average 11.62 µg/100 µl for method I and 11.67µg / 100µL for method II. The third method applied to our mussel samples leads to an extraction of poor yield (7.99 µg/100µl) and quality as the purity of the extracted RNA was low. Indeed the report OD280/OD260 is 1.45, implying sufficient protein contamination to disturb the reverse transcription and PCR (de Silva Gesteira *et al.,* 2003).

RNA yield (criterion2)

For Comparison criterion 2 (RNA yield) involves more significant differences. Several types of similar products based on the same reagents are marketed under proprietary formulations (TRIzol ® and TRIreageant ®, RNAzol ® B ...) (Kingsley *et al.* , 2002). The analysis showed a good extraction for the Methods I and II. There was a significant superiority of the first two methods compared to the method III. Considering criterion 2, the difference between the three methods was less marked. However, the method II was significantly better the method III which had a lower purity. For virological analysis of food it would be better not to use the method III (Weber *et al.*, 1998). The methods I and II are more suitable for this purpose.

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References

Arnal C, Ferre-Aubineau V, Besse B, Mignotte B, Schwartzbrod L, Billaudel S.1999. Comparison of seven RNA extraction methods on stool and shellfish samples prior to hepatitis A virus amplification. Journal of Virological Methods **77**:17-26.

Atmar RL, Neill FH, Romalde JL, Le Guyader F, Woodley CM, Metcalf TG, Estes MK. 1995. Detection of Norwalk virus and hepatitis A virus in shellfish tissues with the PCR. Applied and Enviromental Microbiology **61**,3014-3018.

Bianchi S, Vecchio AD, Vilariño ML, Romalde JL. 2011. Evaluation of different RNA-extraction kits for sensitive detection of hepatitis A virus in strawberry samples. Food microbiology **28**, 38-42.

Boom R, Sor CJ, Salilmans MMM, Jansen CL, Wertheim-Van Dillen PME Van der Noordaa J.1990. Rapid and simple method for purification of nucleic acids. Journal of Clinical Microbiology **28**,495–503.

Bosch A. 1998. Human enteric viruses in the water environment: a minireview. International Microbioliology **1**, 191-196.

Bosch A, Sanchez G, Le Guyader F, Vanaclocha H, Haugarreau L Pinto RM. 2001. Human enteric viruses in Coquina clams associated with a large hepatitis A outbreak. Water Science and Technolology **43**, 61-65.

Bouchriti N, Goyal S M.1993. Methods for the concentration and detection of human enteric viruses in shellfish: a review. New Microbiologica **16**, 105-113.

Chomczynski P. 1993. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques **15,** 532-4.

Chomczynski P, Sacchi N. 1987. Analytical Biochemistry **162**, 156-159.

Cook N. 2003. The use of NASBA for the detection of microbial pathogens in food and environmental samples. Journal of Microbiological Methods **53**,165-174.

Gonzalez-Perez I, Armas Cayarga A, García de la Rosa I, Josefina González González Y. 2007. Homemade viral RNA isolation protocol using silica columns: A comparison of four protocols. Analytical biochemistry **360**, 148-150.

Greene J. 1993. Isolation and purification of nucleic acids: In recombinant DNA methodology course, Bio-tract 2 Fall session **4,** 1-4.

Jiménez-Clavero M A, Ley V, Gómez N, Sáiz JC. 2006. Detection of enteroviruses. In Food-Borne Pathogens p. 153-169. Humana Press.

Karamoko Y, Ibenyassine K, Ait Mhand R, Idaomar M, Ennaji MM.2005. Adenovirus detection in shellfish and urban sewage in Morocco (Casablanca region) by the polymerase chain reaction. Journal of Virological Methods **126**, 135–137

Kingsley DH, Meade GK, Richards GP .2002. Detection of both hepatitis A virus and Norwalk-like virus in imported clams associated with food-borne illness. Applied and Environmental Microbiology **68**,3914-3918.

Le Guyader FS Atmar RL .2007. Viruses in shellfish. Perspectives in Medical Virology **17**, 205-226.

Richards GP .1999. Limitations of molecular biological techniques for assessing the virological safety of foods. Journal of Food Protection **62**,691-697.

Santos N, Gouvea V. 1994. Improved method for purification of viral RNA from fecal specimens for rotavirus detection. Journal of Virological Methods **46**, 11-21.

de Silva Gesteira A, Micheli F, Ferreira CF, de Mattos Cascardo, JC. 2003. Isolation and purification of functional total RNA from different organs of cacao tree during its interaction with the pathogen Crinipellis perniciosa.Biotechniques **35**, 494-501.

Weber K, Bolander ME, Sarkar G. 1998. PIG-B: a homemade monophasic cocktail for the extraction of RNA. Molecular Biotechnology **9**, 73-77.