Laboratory challenges for the detection of norovirus in environmental samples

Susana Guix (susanaguix@ub.edu)

"Outbreaks of norovirus gastroenteritis: An emergent health issue"

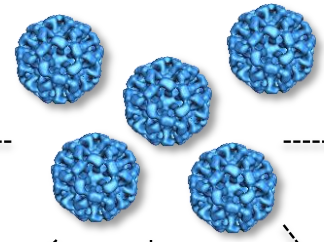
SCIENTIFIC SESSION

Monday, 23 November 2015
From 9:00 to 14:15 hours

VIRUS ENTÈRICS
UNIVERSITAT DE BARCELONA
INSA UB
Instituto de Salud Carlos III
AQUAVALENS

Environmental Contamination

Fecally contaminated water



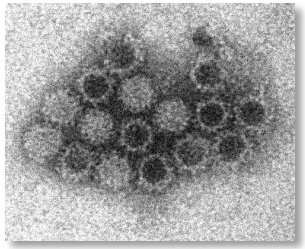
Food items handled by an infected person

Fresh produce contaminated by irrigation water

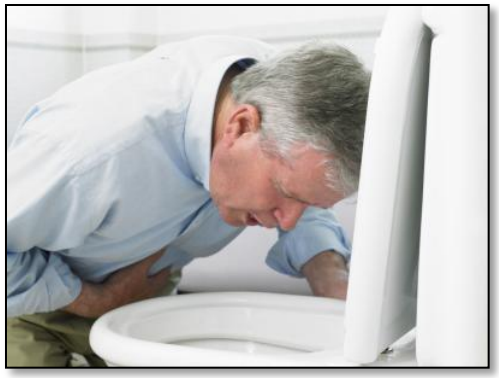
Shellfish

Main Food and Waterborne Viruses

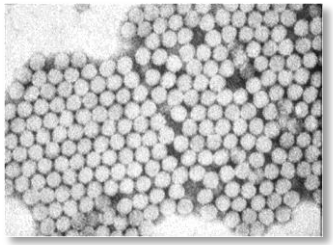
Norovirus (NoV)



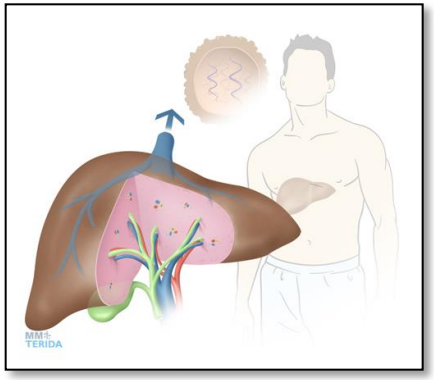
Acute Gastroenteritis



Hepatitis A Virus (HAV)



Acute Hepatitis



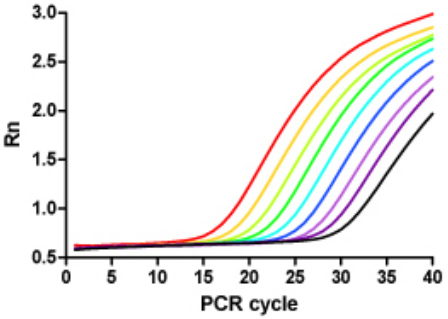
Virus Detection on Food and Water

Main challenges:

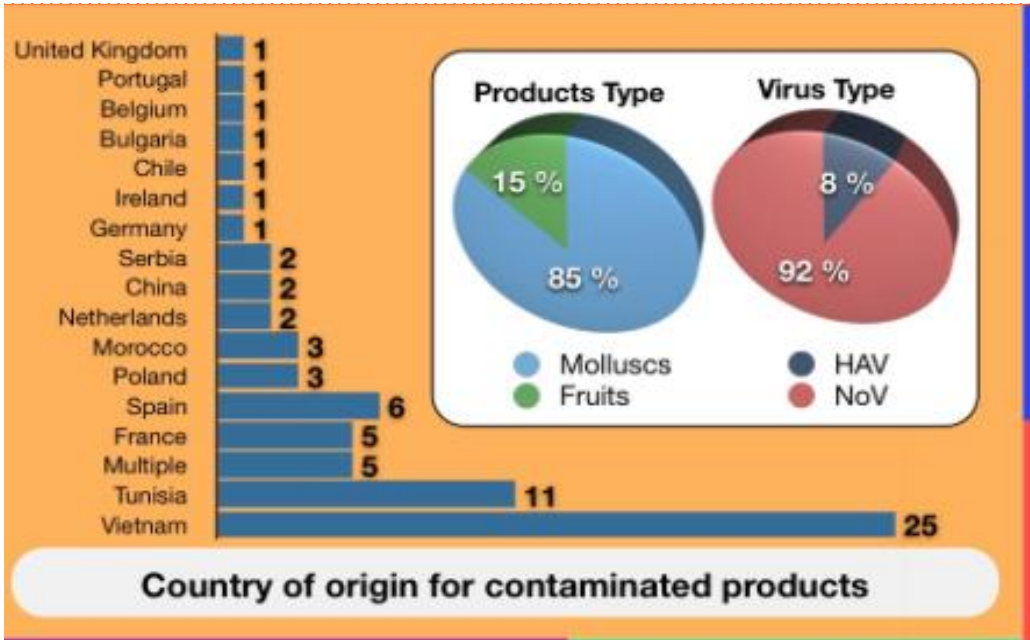
- Low levels of viral contamination
- Presence of inhibitory substances

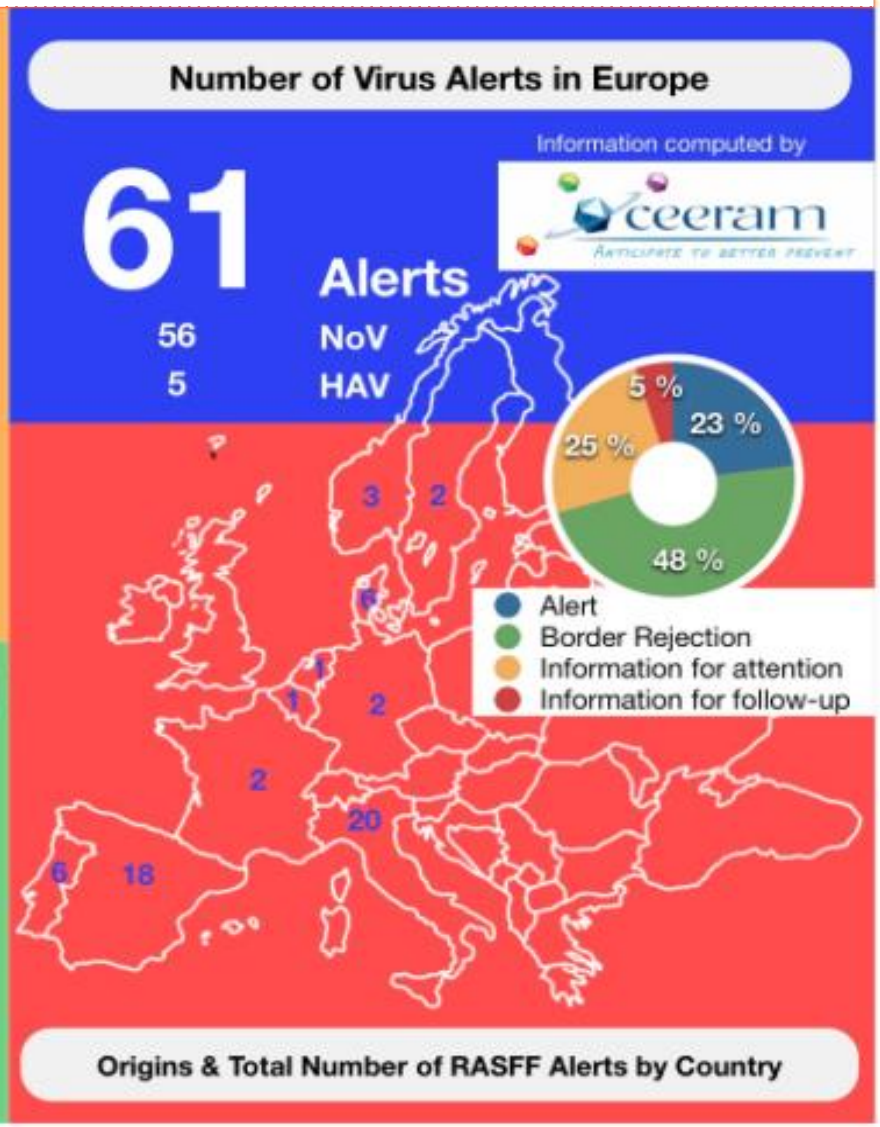
ISO/TS 15216

Microbiology of food and animal feeding stuffs -- Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR -- Part 1: Method for quantitative determination



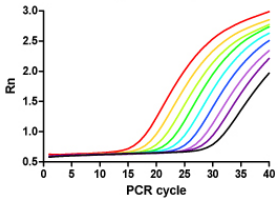
Virus Alerts in Europe in 2014
Rapid Alert System for Food and Feed (RASFF)





Source : RASFF
https://webgate.ec.europa.eu/rasff-window/portal/?event=SearchByKeyword&NewSearch=1&Keywords=norovirus
https://webgate.ec.europa.eu/rasff-window/portal/?event=SearchByKeyword&NewSearch=1&Keywords=hepatitis

Molecular Methods Shortcomings

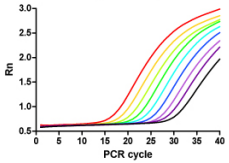


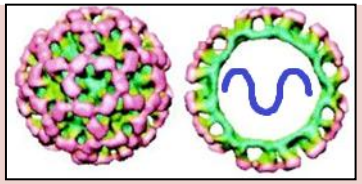
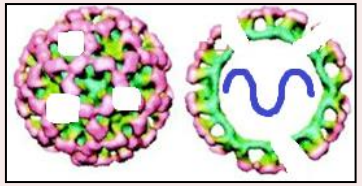
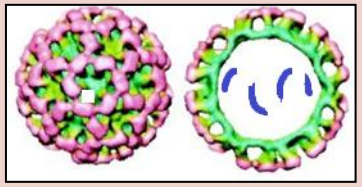
A. Lack of correlation with infectious titers:

- A positive RTqPCR signal may not always correlate with the presence of infectious virus.
- Lack of cell culture methods to quantify infectious virus titers for most foodborne viruses.

B. Elevated cost of the analyses

Molecular Methods Detect Both Infectious and Non-infectious Viruses



		RTqPCR	Cell Culture
Undamaged virions		+	+
Virions with damaged capsid		+	-
Virions with damaged genome		+	-

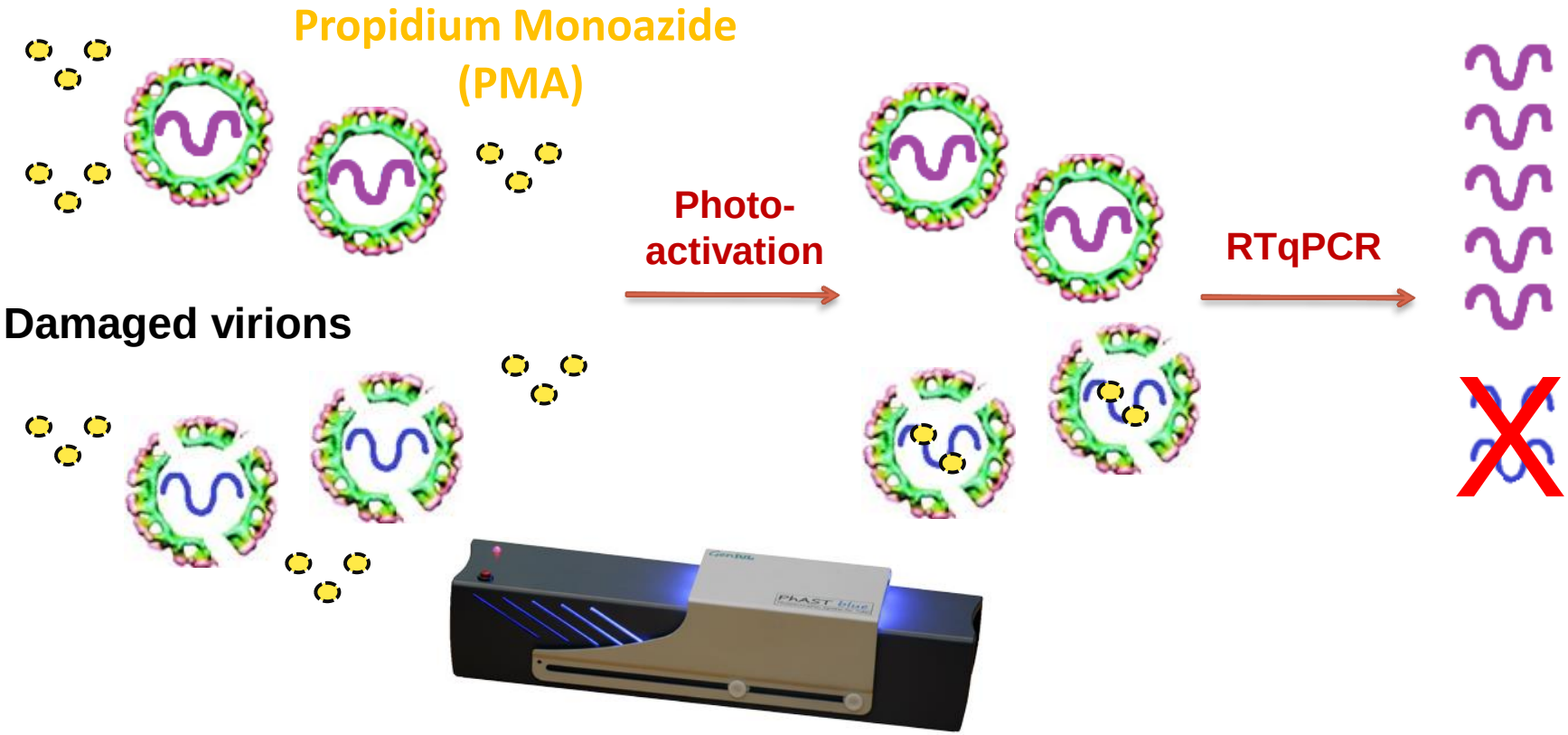
Objectives

1) Optimize molecular procedures to get a better correlation with infectivity

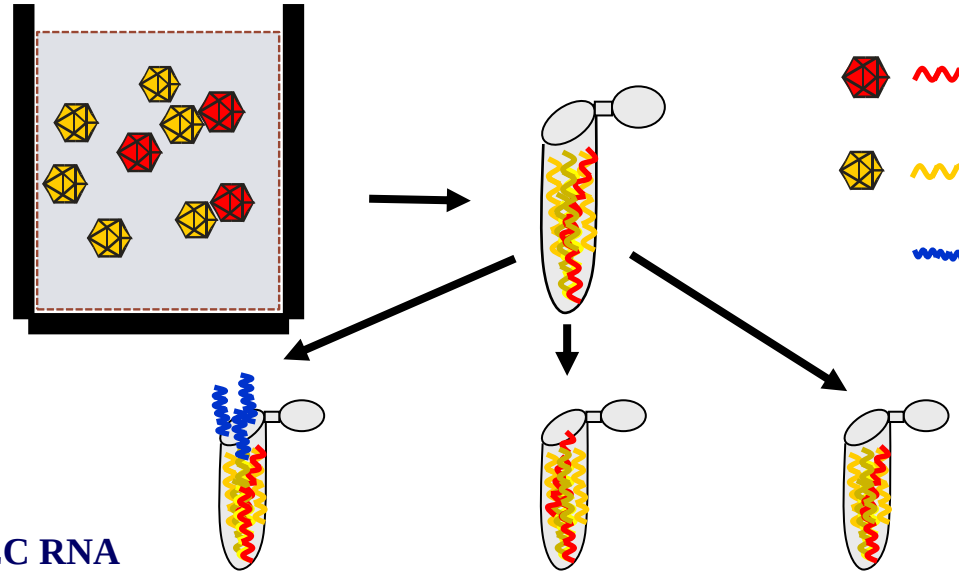
- The correlation between infectivity and genome copies was evaluated by performing pretreatments with intercalating dyes such as PMA, prior to RTqPCR.
- Different matrices were evaluated: (a) shellfish and (b) water samples

2) Reduce the cost of RTqPCR analysis by setting up a multiplex assay

1. Use of PMA-RTqPCR assays



Controls and Standardization of RTqPCR

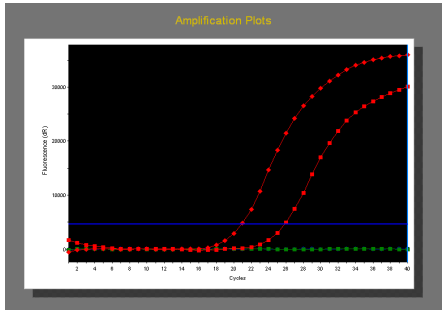


Target virus

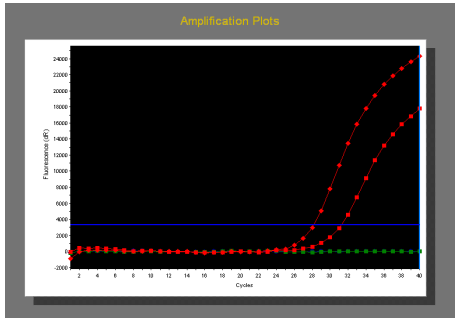
Mengovirus

Target external control RNA (EC)

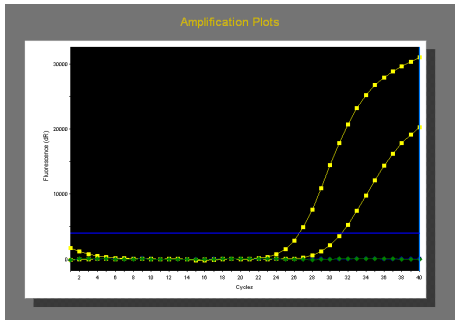
Target virus + EC RNA RT-PCR Efficiency



Target virus Quantitation



Mengo Quantitation RNA Extraction Efficiency

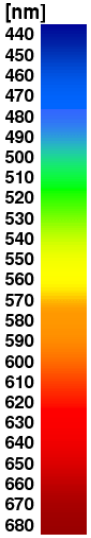


Virus Quantitation Accuracy

Costafreda *et al*, 2006 (*AEM*); Pintó *et al*, 2009 (AEM)

2. RTqPCR cost reduction by multiplexing

Target	Fluorophors	Abs peak (nm)	Em peak (nm)
HAV	FAM	492	520
Mengo	VIC	538	554
NoV GI	Texas Red	595	615
NoV GII	Atto647N	644	669



Monoplex plate layout to analyze 1 sample (65 wells)

HAV | GI | GII | Mengo

Sample | Standards and Controls

Sample D	Sample D	Sample D	Sample D		dsDNA D	dsDNA D	dsDNA D	dsDNA D	dsDNA D	dsDNA D	RNA Mengo D
Sample D	Sample D	Sample D	Sample (-1)		dsDNA (-1)	dsDNA (-1)	dsDNA (-1)	dsDNA (-1)	dsDNA (-1)	dsDNA (-1)	RNA Mengo (-1)
Sample (-1)	Sample (-1)	Sample (-1)			dsDNA (-2)	dsDNA (-2)	dsDNA (-2)	dsDNA (-2)	dsDNA (-2)	dsDNA (-2)	RNA Mengo (-2)
Sample (-1)	Sample (-1)	Sample (-1)			dsDNA (-3)	dsDNA (-3)	dsDNA (-3)	dsDNA (-3)	dsDNA (-3)	dsDNA (-3)	RNA Mengo (-3)
Sample D +EC RNA	Sample D +EC RNA	Sample D +EC RNA			dsDNA (-4)	dsDNA (-4)	dsDNA (-4)	dsDNA (-4)	dsDNA (-4)	dsDNA (-4)	
Sample (-1) +EC RNA	Sample (-1) +EC RNA	Sample (-1) +EC RNA									
H_2O +EC RNA	H_2O +EC RNA	H_2O +EC RNA			Neg Ext		Neg Ext		Neg Ext		Neg Ext
					Neg qRT-PCR		Neg qRT-PCR		Neg qRT-PCR		Neg qRT-PCR

Multiplex plate layout to analyze 1 sample (43 wells)

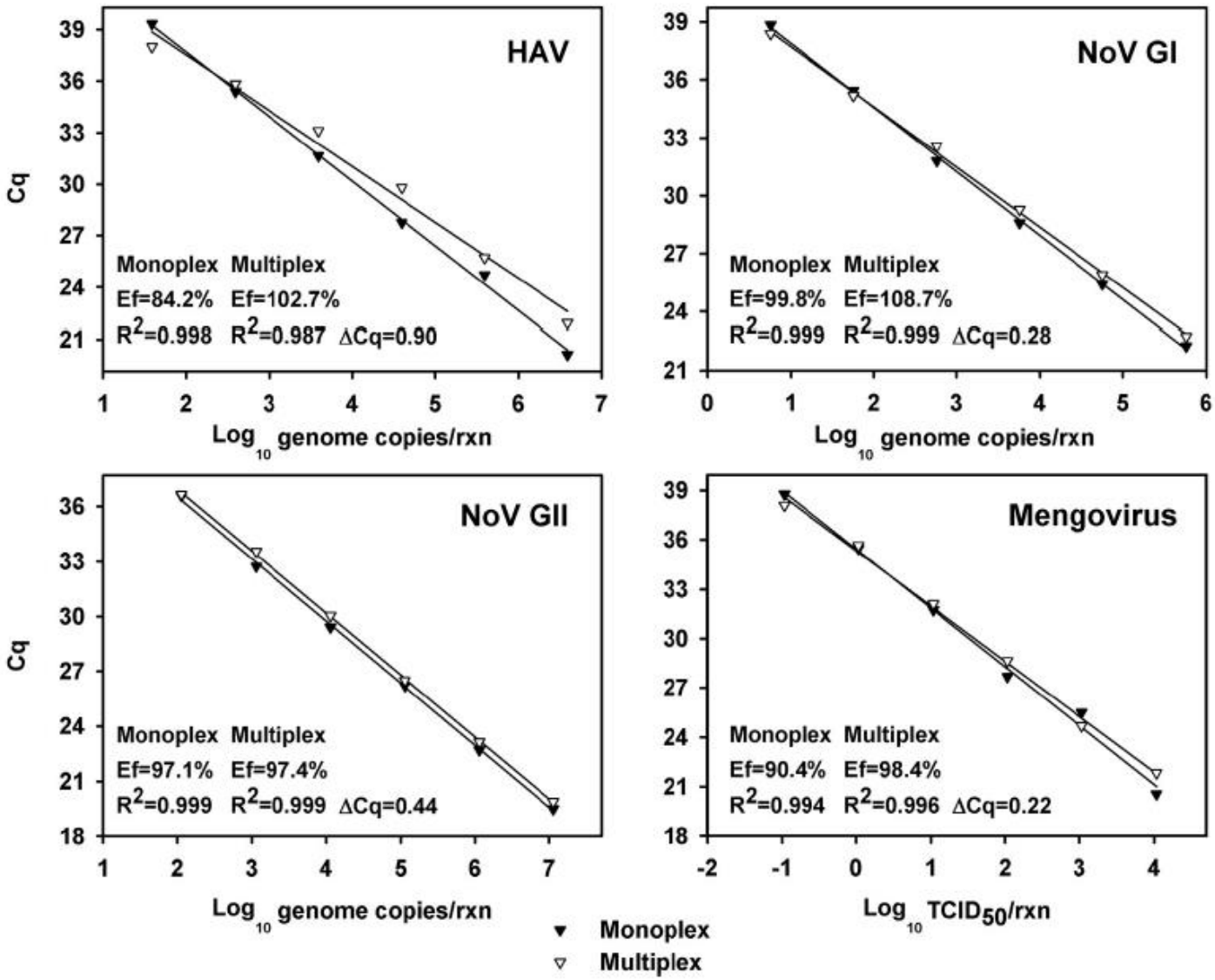
HAV | GI | GII | Mengo | Multiplex

Sample | **Standards and Controls**

Sample D	Neg Ext				dsDNA D	dsDNA D	dsDNA D	dsDNA D	dsDNA D	dsDNA D	RNA Mengo D
Sample D	Neg qRT-PCR				dsDNA (-1)	dsDNA (-1)	dsDNA (-1)	dsDNA (-1)	dsDNA (-1)	dsDNA (-1)	RNA Mengo (-1)
Sample (-1)									dsDNA (-2)	dsDNA (-2)	RNA Mengo (-2)
Sample (-1)										dsDNA (-3)	RNA Mengo (-3)
Sample D +EC RNA										dsDNA (-4)	
Sample (-1) +EC RNA											
H_2O +EC RNA											

6 samples /plate

Optimized assay on viral RNAs



Fuentes *et al*, 2014 (*Food Microbiology*)

Shellfish samples

Sample	Virus	MONOPLEX	MULTIPLEX
Oysters (UVEVV07/10)	HAV	ND	ND
	GI	1.94 ± 0.14	1.60
	GII	3.96 ± 0.05	3.26 ± 0.01
Oysters (UVEVV126/11)	HAV	ND	ND
	GI	2.07	ND
	GII	2.54	1.94
Coquina clams (Val01/08)	HAV	3.14 ± 0.16	2.82 ± 0.38
	GI	2.08 ± 0.21	1.76
	GII	2.80 ± 0.28	2.31 ± 0.28
Coquina clams (Val02/08)	HAV	3.91 ± 0.00	3.69 ± 0.02
	GI	2.22 ± 0.09	1.76
	GII	3.34 ± 0.03	2.31 ± 0.28
Coquina clams (Val03/08)	HAV	3.55 ± 0.02	3.25 ± 0.44
	GI	2.38 ± 0.22	1.75
	GII	3.77 ± 0.03	3.42 ± 0.18
Coquina clams (Val04/08)	HAV	3.53 ± 0.07	3.61 ± 0.27
	GI	2.68 ± 0.59	1.78 ± 0.00
	GII	3.33 ± 0.01	2.52 ±0.01
Coquina clams (Val05/08)	HAV	4.28 ± 0.03	3.62 ± 0.02
	GI	2.10 ± 0.24	2.10 ± 0.16
	GII	3.63 ± 0.00	2.88 ± 0.01

Fuentes *et al*, 2014 (*Food Microbiology*)

ND: Not Detected

Mean ± standard error of the Log_{10} genome copies of HAV and NoV GI and GII per g of hepatopancreas

Differences in Log_{10} titers between monoplex and multiplex

Log_{10} Viral Load	HAV	GI	GII		Average (Mean ± SEM)
6	0,49	-	0,03		0,23 ± 0,26
5	0,26 ± 0,15	-	0,48 ± 0,02		0,35 ± 0,09
4	0,14	0,48 ± 0,18	0,71 ± 0,01		0,49 ± 0,12
3	-	0,17 ± 0,30	0,70		0,34 ± 0,25
2	-	-	0,45 ± 0,13		0,45 ± 0,13
1	0,57	0,34	0,82 ± 0,24		0,64 ± 0,15
0	-	-	0,39 ± 0,22		0,39 ± 0,22
Average	**0,32 ± 0,10**	**0,37 ± 0,13**	**0,51 ± 0,08**		**0,43 ± 0,06**

Fuentes *et al*, 2014 (*Food Microbiology*)

In Summary

- PMA pretreatment improves correlation with infectious titer, especially for HAV. PMA pretreatment may be easily applied in water samples, but sample dilution is required for PMA to be effective on shellfish homogenates. It may be problematic when levels of viral contamination are low.
- PMA pretreatments may be helpful when interpreting levels of viral contamination used for microbiological quantitative risk assessment studies.
- The multiplex assay represents a time-saving and cost-effective tool to detect and accurately quantify HAV, NoV GI and GII in selected commodities.

Acknowledgements

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Thank you for your attention