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TRANSMISSION ROUTES OF NOROVIRUSES, EMERGING HUMAN PATHOGENS IN FOOD

"NORISK"

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FINAL REPORT

TRANSMISSION ROUTES OF NOROVIRUSES, EMERGING HUMAN PATHOGENS IN FOOD

"NORISK"

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ACRONYMS, ABBREVIATIONS AND UNITS

ARSIA	Association Régionale de Santé et d'Identification Animales
BSA	bovine serum albumin
Bp	Base pairs
°C	Celsius degree
C1	Coordinator – partner 1 (ULg –Virology)
cDNA	complementary DNA
CEFAS	Centre for Environment, Fisheries & Aquaculture Science
CEN	European Committee for Standardization
CCV	Canine calicivirus
CSS	Conseil Supérieur de la Santé
Ct	Threshold cycle
Det.	Detection
DGZ	Dierengezondheidszorg Vlaanderen
DNA	Desoxyribonucleic acid
EFSA	European Food Safety Agency
FAM	6-carboxyfluorescein
FASFC	Federal Agency for the Safety of the Food Chain (Belgium)
FCV	Feline calicivirus
Fig	figure
GGI	Genogroup 1
GGII	Genogroup 2
GIGA	Groupe Interdisciplinaire de Génoprotéomique Appliquée
h	hour
H_2O	dihydrogen monoxid (water)
HAV	Hepatitis A virus
HGR	Hoge Gezondheidsraad
IAC	Internal Amplification Control
IC	internal control
IPH	Institute of Public Health (Belgium)
ISP	Institut de la Santé Publique
INTA	Instituto Nacional de Tecnología Agropecuaria
Kb	Kilobase
KUL	Katholieke Universiteit Leuven
mM	Millimolar
MMX	mastermix
MNV-1	murine norovirus 1
MW	Molecular Weight
NCBI	National Centre for Biotechnology Information
Ng	Nanogram
nM	Nanomolar
NRL-FBO	National Reference Laboratory of food-borne outbreaks
NRL-VTI	Nationaal Referentie Laboratorium van voedseltoxi-infecties
NTC	No target control
NV(s)	Norovirus(es)
ORF	Open Reading Frame
P2	Partner 2 - Ugent
P3	Partner 3 - IPH
P4	Partner 4 – ILVO
P5	Partner 5 – ULg –Food microbiology
PBS	phosphate buffered saline
PCR	Polymerisation chain reaction

R^2	Correlation coefficient
Ref	reference
RNA	Ribonucleic acid
RIVM	Rijksinstituut voor Volksgezondheid en Milieu
RT-PCR	Reverse transcriptase polymerase chain reaction
ssDNA	single stranded DNA
SV(s)	sapoviruses
T^+	internal positive control
TAMRA	6-carboxy-tetramethylrhodamine
UCL	Université Catholique de Louvain
UGent	University of Ghent
ULg	University of Liège
UNG	uracil-N-glycosylase
WG	Working group
µl	Microliter
%	percent

PROJECT SUMMARY

CONTEXT

Noroviruses (NV) are among the most important causes of gastroenteritis in adults worldwide and often occur as outbreaks. In the Netherlands, the Public Health Institute investigated 153 outbreaks of acute gastroenteritis between 1994 and 1999. Of those outbreaks 17% were considered food-borne and 76% were presumptively caused by NV. Bivalve shellfish are notorious as a source of food-borne viral infections, because filter-feeding bivalves can concentrate viruses. Several other foods have been implicated as vehicles of transmission (fruits, vegetables, sandwiches) contaminated by contact with polluted water in the growing area or during processing or by unhygienic handling during distribution or final preparation. Furthermore, NVs are present in several animal species, raising important questions about zoonotic transmission and potential animal reservoir.

OBJECTIVES

- Elaboration, optimization and evaluation of a real-time PCR format and determination of its specificity, sensitivity and robustness.
- Evaluation of the effectiveness of several virus concentration / viral RNA extraction and purification protocols from a variety of food matrices and elaboration of an appropriate extraction procedure in fresh produce/ready-to-eat foods.
- Development and implementation of a standard protocol with establishment of appropriate controls for routine detection of NVs in food stuffs (seafood and fresh products).
- Elucidation of transmission routes (zoonosis hypothesis) through molecular tracing, with a global view on NV strains circulating among human, animal and also in food.
- Tracing of outbreaks: scenario for coupling clinical data from NV outbreaks to their food-borne cause and risk profiling.
- Development of a risk profile.
- Tracing of the genetic evolution of NVs: genetic profiles and emerging of recombinants.

WORKPLAN

- **Methods of analysis**
  This part was performed as in the initial planning and will be implemented during the second part of the project

  ° Real-time PCR human, animal and food samples (primer selection, probes and SYBR Green, quantification with Murine NV and/or Feline Calicivirus)
  ° Extraction – concentration methods (water, ready to eat food, fruits, shellfish)

- **Virus evolution**
  This part was performed as in the initial planning and will be implemented during the second part of the project

  ° Genotyping : NVs in human samples, animals samples, shellfish, screening of food samples at retail, processing units, primary production for NV contamination
  ° Recombinants : NVs in human samples, animals samples, shellfish

- **Risk profiling**
  This part will be implemented during the second part of the project
- **Development of network**
  This part has been started and will be implemented during the second part of the project.

RESULTS-CONCLUSIONS

The methods of analyses for the detection of NV in different food matrices will be optimized and validated.

A. Real time RT-PCR protocols have been evaluated for detection of GGI and GGII NVs. The use of the Taqman Universal Mastermix has been privileged in combination with the CEN/TC/WG6/TAG4 primers and probes. The methods were optimized using pGI and pGII plasmids as standard instead of single stranded DNA fragments to prevent contamination. Optical adhesive films were preferred to seal the 96-well plates to limit contaminations. The real-time PCR protocol for the detection of MNV-1 designed by Baert et al., 2008 (2) was shown to be appropriate for the detection of MNV-1. All singleplex assays were successfully tested on two different thermocyclers (ABI Prism® SDS 7000 and Roche Lightcycler LC480). Analysis of the detection limit of the 3 individual assays showed that a minimum of 10 copies of the pGI/pGII/p20.3 plasmids – containing primers-probe binding sites of respectively GGI and GGII NVs and MNV-1 – were consistently detected at mean Ct values of respectively 37.38/38.02/35.11.

B. All optimized singleplex real-time PCR assays were combined into one multiplex assay and, when equally mixed amounts of the pGI, pGII and p20.3 plasmids were detected with the multiplex assay, only a negligible loss in sensitivity was noticed in comparison to the singleplex reactions.

When pGI, pGII and p20.3 plasmids were mixed in different concentrations, a mutual competitive effect was noticeable between the individual GGI and GGII reactions within the multiplex assay. This competitive effect became clear when a 2 log excess (10^5 / 10^3 copies and 10^3 / 10 copies) was present between the 2 targets (pGI/pGII), resulting in Ct-shifts between 1.8 and 5.6 Ct. Moreover, when a 4 log excess (10^5 and 10 copies) was present between the 2 targets (pGI/pGII), the target with the lowest concentration could not be detected (Ct>50).

The effect of the MNV-1 reaction on the GGI and GGII reactions within the multiplex assay was limited when pGI or pGII were solitarily present. However, the presence of 10^3 and 10^5 copies of p20.3 did cause Ct-shifts when a 2 log concentration difference between GI and GII was present.

This observation showed the limits of the multiplex assay for the detection of low amounts of one NV genotype (GGI/GGII) in the presence of high amounts of another NV genotype (GGII/GGI) in the same sample.

These results also indicated that the use of the MNV-1 reaction as PCR internal amplification control (IAC) is achievable. To avoid any competitive effects and to avoid the loss of the quantitative properties of the multiplex assay (especially when detecting low virus concentrations), no more than 10^2 to 10^3 copies of plasmid p20.3 should be added to the real-time PCR reaction as IAC when detecting GI/GII NoVs.

C. Specificity and sensitivity of the multiplex assay was analyzed by testing 16 clinical samples, a Norovirus RNA reference panel and 7 alternative viruses. All samples previously found positive for GGI or GGII NVs were also detected in the respective GI or GII PCR assays within the multiplex PCR. All tested genotypes present in the Norovirus reference panel were specifically detected. No cross amplification between the GI and GII genogroups was noticed. Negative samples and all alternative virus types tested negative.

The developed multiplex real-time RT-PCR assay is a specific and sensitive method for quantification of GGI and GGII NVs. Partner 3 will use and evaluate this assay for detection of NVs in clinical/food samples in case of suspected foodborne NV outbreaks.

Further development of a method for the detection of NVs in food matrices will include the development/optimization of the sample preparation: different protocols for the virus/RNA extraction on different food matrices (fresh produce/ready-to-eat foods) will be compared and evaluated. In this study, products will be spiked with MNV-1 and the extraction efficiency will be analysed by the developed real-time RT-PCR method.

The CEN WG6 TAG4 protocol has been tested on different shellfish matrices namely mussels and oysters during the first part of the project. To limit environmental contamination hampering the project at its start, a novel internal control was developed to avoid the use of a NV sequence in the reactions as recommended by the CEN. Detection limits of 35 particles per reaction and 25-250 particles per reaction for GGI and GII respectively were determined based on synthetic RNA. Ring-tests for NV

detection in shellfish have been organized between the CEN members and situated the P5 laboratory within the best scoring. Still, the detection of GGI was less effective in the food matrices and samples tested in duplicate gave opposite results. The use of a different commercial mastermix especially developed for low copy RNA detection could raise the sensibility of the reaction. Threshold cycle values for the detection of GGI and GGII in shellfish were extremely high compared to those observed in stool samples indicating viral contamination of shellfish to be very low. In the next phase the sample preparation and the viral extraction method in shellfish will be optimized. An appropriate extraction method will not only improve the detection of NV but also could make amplification for genotyping possible hampered up to date by a lack of genetic material in samples.

NV and calicivirus strains were detected from animal fecal samples collected at the beginning of the project. Most of bovine NVs detected corresponded to the GGIII.2 Newbury strain. The identification of several natural recombinant strains GIII.1/GIII.2 confirms that not only human noroviruses are capable of recombination. Even if up to date there is no clear evidence that cattle can be infected by human noroviruses, this finding could maintain the question of the zoonotic potential of animal NV. Recombination events could engender novel strains capable of crossing the species barrier. This scenario would be more susceptible to occur in countries where humans and animals with high human and cattle densities like Belgium. The detection of NV and SV closely related to human NV and SV strains in pigs fears for this potential zoonotic risk. Pigs have been shown to be experimentally capable of being infected by human NV but this statement could not be confirmed in field studies until now. Recombination between human and porcine NV and SV has not yet been described but should not be excluded assuming their genetic relatedness.
Genotyping of the detected NV strains in the different matrices is important to understand transmission routes of NV. All positive clinical samples from outbreaks in 2007 provided by the IPH were GGII.4 variants 2006a and 2006b. These results would confirm other reports that, since their emergence in 2006, describe these variants as the most circulating strains worldwide (only the denomination changes over the continents; they are known as Laurens and Minerva in the USA, v4 and v6 in the UK). The two variants were thought to co-circulate in the same proportions but GII.4 2006b seems more prevalent in most European countries for outbreaks in season 2007-2008. Unfortunately the lack of positive samples in this study did not allow us to confirm this on a Belgian level. For 2008-2009 this trend was not observed, GII.4 2006 variants were still implicated in outbreaks but other norovirus genotypes and genogroups were detected in stools from outbreaks and sporadic cases of gastro-enteritis. One sample seemed to be co-infected by two different genogroups (GGII and GGIV). As co-infection could enhance recombination events, the sample will be further investigated to find out if there is evidence of recombination. One sporadic gastro-enteritis case showed the presence of a sapovirus GI.2, this result could not be included in the risk analysis study for Belgium because the sample originated from a bordering region in France. No other sample was positive for the genus sapovirus, to our knowledge neither outbreak nor sporadic case of gastroenteritis could be linked to the presence of sapovirus in Belgium although they have been detected in several bordering countries like France and The Netherlands.
The characterization and the study of recombinant NVs require sequences that cover the ORF1/ORF2 junction and the whole capsid gene sequence. For six clinical samples a fragment covering both these regions could be amplified, so we could assure that the sequences of the polymerase and the capsid region both issued from the same genome and that these strains did not undergo recombination. Unfortunately we did not succeed yet in amplifying a large fragment of the potential recombinant GIIb/GII.3 strains UCL5 and 6. The sequences from the polymerase and the capsid regions of these samples did not cluster in the same genotype. A fragment of approximately 1000 bp covering the ORF1-ORF2 junction was amplified for UCL5. The Simplot analysis with the putative parental strains indicated the breakpoint to be at the junction between the polymerase and the capsid. This confirms what was observed before for other NV and SV recombinant strains.
Positive shellfish and food samples provided by P5 and P3 respectively were amplified for sequence analysis. A lack of material made it impossible to amplify enough exploitable DNA. The following phase will be fully consecrated to this purpose. Food samples were linked with outbreaks and even that there was not enough RNA in the samples. Inhibition in this kind of matrices is very important and the extraction method is a crucial step for the detection of viruses.

Since the start of the reporting, the causative agent remains unknown in 20 to 50% of the reported food-borne outbreaks in Belgium. NV is suspected to be an important cause of food-borne outbreaks and could be responsible for a large part of these unknown cases. However up to now no robust extraction and detection system for the detection of this virus is available for routine analyses of different kinds of foodstuffs, neither there is an international approved isolation and detection method for NV in different kind of foods. Furthermore the procedures described in literature are not suitable for a routine analysis. Moreover, in most of the cases no fecal samples of the patient are taken and in some cases there remain no leftovers of the food. So it is difficult to find the epidemiological link to trace back the contaminated food which has been the source of the infection. Also NV infections are underestimated because the symptoms are normally self-limiting in 24 h and complications are rare. Because of these shortcomings a better protocol was worked out with the doctors of the health inspections to send us the faecal material. Faecal material is much easier to analyze than food, because of the higher concentration of virus particles and already al lot of standard RNA extraction protocols available. However, it was also possible to detect NV in different kind of foods by the procedure described by Baert et al. 2006. Now we are waiting for the optimized protocol of partner 4 to change our food extraction protocol to have an overall better sensitivity of the procedure.

RECOMMENDATIONS

The Norisk network was already able to setup and apply a diagnostic procedure of NV detection of food matrices and human samples. The diagnostic procedures allowed the identification of several outbreaks of gastroenteritis. The application of this procedure allowed the identification of NV as the first cause of food-borne gastroenteritis in Belgium in 2007.
Therefore public health should be concerned by this diagnostic figure in Belgium and instructions should be given to professionals in order to reduce the risk of food contaminations and inter-human dissemination of the infection.
The recommendations arisen from Norisk scientific work are being distributed to the scientific and medical communities through the participation of the Norisk partners to several committees and working parties. All partners are members of the working group of the Belgian *Conseil Supérieur de la Santé (CSS) – Hogegezondheidsraad (HGR)* to study virus transmission by food. Partners 1 and 2 both participate in the European Network for Environmental and Food Virology (COST Action 929). Partners 3 and 5 are National Reference Laboratories of foodborne outbreaks and viral contaminants of shellfish respectively.

I. CONTEXT

Noroviruses (NV - previously known as Norwalk-like caliciviruses) are among the most important causes of gastroenteritis in adults and often occur as outbreaks which may be foodborne. Furthermore, NV, thought to be restricted to human, are present in bovine and porcine species, raising important questions about zoonotic transmission and potential animal reservoir. The aim of this project is to elucidate the transmission routes of NV to human while increasing food safety for the consumer and improving public health. For this reason the project initially focuses on the development of appropriate real-time RT-PCR for either detection or genotypic analysis of NVs. Subsequently the isolated strains will be studied to elucidate the recombination phenomenon and mechanisms, and animal NVs regarding zoonotic hypothesis. In addition, data on the importance of NVs in the food chain will be added.

II. OBJECTIVES

The objectives of this project consider

1) The NVs RNA detection methodology: elaboration, optimization and evaluation of a real-time PCR format and determination of specificity, sensitivity and robustness. Two protocols will be developed. A real-time PCR protocol directed to detection of the acknowledged GGI and GGII strains involved in outbreaks to be used in the frame of control and surveillance by food authorities and food business operators to verify their products and production process. Another real-time RT PCR protocol directed towards a wide diversity of NV genogroups (including newly reported animal associated NV) to be used for research purposes to establish transmission routes and document circulating strains in the environment.
2) The sample preparation method: to evaluate the effectiveness of several virus concentration / viral RNA extraction and purification protocols from a variety of food matrices in particular seafood and with emphasis on elaboration of an appropriate extraction procedure in fresh produce/ready-to-eat foods.
3) The routine detection of NVs in food stuffs (seafood and fresh products): to develop and implement a standard protocol with establishment of appropriate controls for rapid screening of foods for the presence of NVs in accordance with the guidelines for officially approved analysis and harmonization and to generate information on the prevalence of NV strains in foods at retail, products and production processes under the control of food business operators and the primary production.
4) Elucidation of transmission routes (zoonosis hypothesis) through molecular tracing, with a global view on NV strains circulating among human, animal and also in food.
5) The tracing of outbreaks: scenario for coupling clinical data from NV outbreaks to their foodborne cause and risk evaluation.
6) The development of a risk profile on NV present in the food chain and animal species (strain types circulating, potential animal reservoir, zoonose, definition and incidence in at risk foods, link to epidemiological information).
7) Tracing of the genetic evolution of NVs: genetic profiles and emerging of recombinants.

III. METHODOLOGY

Work package 1: Methods of analysis

Optimization and validation of real-time RT-PCR methods for detection of human genotypes (GGI and GGII) of NVs and for MNV-1 (P4, with input of P2 and P3).

A. Optimization of singleplex real-time PCR assays for detection of GGI and GGII NVs and for MNV-1.

All optimization steps of the singleplex assays were performed on a ABI Prism® SDS 7000 real-time PCR system under the following conditions: incubation at 50°C for 2 min to activate UNG, initial denaturation at 95°C for 10 min, followed by 50 cycles of amplification with denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Amplification data were collected and analysed with the instruments' software.

1. Optimization singleplex real-time PCR assays for detection of GGI & GGII NVs.

1.1. Comparison of different PCR mastermixes

The use of separate reagents (Table 1) has been compared versus the use of commercial (prepared) mastermixes (Table 2). Single stranded DNA fragments described in point 1.3 were used as 10-fold diluted real-time PCR standard series (10^7 – 10 copies).

Table 1: Set-up real-time PCR reaction mix using separate reagents.

Product	Firm	Final concentration
10x PCRGold buffer	Applied Biosystems	1x
MgCl2	Applied Biosystems	1-4mM
Rox Reference Dye	Invitrogen	1x
dNTP's	GE Healthcare	1µM
AmpliTaq Gold Polymerase	Applied Biosystems	1,25u

Table 2: Different commercial mastermixes.

Product	Firm	Final concentration
TaqMan Universal MMX	Applied Biosystems	1x
GeneExpression MMX	Applied Biosystems	1x
BlueRox MMX	Westburg	1x

1.2. Comparison of different primers-probe sets

Two described primer-probe sets were compared; one designed by Jothikumar et al., 2005 (Table 3), the other designed by the CEN/TC/WG6/TAG4 research group (Table 4). ssDNA fragments described in point 1.3 were used as 10-fold diluted real-time PCR standard series.

Table 3: Primers-probe sets described by Jothikumar et al., 2005 (1).

Genogroup	Primer/ Probe	Sequence (5' – 3')	Position	Final concentration	Fluorophore(5')[c] / Quencher (3')[d]
GGI	JJV1F	GCCATGTTCCGITGGATG	5282-5299[a]	250 nM	
	JJV1R	TCCTTAGACGCCATCATCAT	5377-5358[a]	250 nM	
	JJV1P	TGTGGACAGGAGATCGCAATCTC	5319-5341[a]	100 nM	FAM/BHQ-1
GGII	JJV2F	CAAGAGTCAATGTTTAGGTGGATGAG	5003-5028[b]	250 nM	
	COG2R	TCGACGCCATCTTCATTCACA	5100-5080[b]	250 nM	
	RING2-TP	TGGGAGGGCGATCGCAATCT	5048-5067[b]	100 nM	YY/BHQ-1

Table 4: Primers-probe sets described by the CEN/TC/WG6/TAG4 research group.

Genogroup	Primer/ Probe	Sequence (5' – 3')	Position	Final concentration	Fluorophore(5')[c]/ Quencher (3')[d]
GGI	QNIF4	CGCTGGATGCGNTTCCAT	5291-5308[a]	500 nM	
	NV1LCR	CCTTAGACGCCATCATCATTTAC	5354-5376[a]	900 nM	
	NVGG1p	TGGACAGGAGAYCGCRATCT	5321-5340[a]	100 nM	FAM/BHQ-1
GGII	QNIF2	ATGTTCAGRTGGATGAGRTTCTCWGA	5012-5038[b]	500 nM	
	COG2R	TCGACGCCATCTTCATTCACA	5100-5080[b]	900 nM	
	QNIFS	AGCACGTGGGAGGGCGATCG	5042-5061[b]	250 nM	YY or TR/BHQ-1

[a] Position in M87661-ref genome, [b] Position in X86557-ref genome, [c] YY: Yakima Yellow; TR: Texas Red, [d] BHQ: Black Hole Quencher

(1) Jothikumar, N., Lowther, J. A., Henshilwood, K., Lees, D. N., Hill, V. R. & Vinje, J. (2005). Rapid and sensitive detection of noroviruses by using TaqMan-based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples. Applied and Environmental Microbiology, 71, 1870-1875.

1.3. Comparison of different types of template as standard (positive controls).

Both synthetic single stranded DNA-fragments (Eurogentec) covering the primers-probe binding sites and plasmids pGI and pGII containing the cloned target area of the primers-probe binding sites were tested.
The plasmid used as standard in the real-time PCR detection of GGII NVs (pGII) was constructed by Partner C1 in the network by cloning the target region of the CEN-GGII assay into the pGEM-T-Easy vector (Promega). The plasmid used as standard in the real-time PCR detection of GGI NVs (pGI) was constructed by partner P4, by cloning the target region of the GGI real-time PCR assay designed by Jothikumar et al., 2005 in the pMOS Blue vector (Amersham). This target region also covers the primers-probe binding sites of the CEN GGI-assay.

1.4 Two different sealing systems of the 96-well real-time PCR reaction plates were compared:

- MicroAmp™ 96- & 384-Well Optical Adhesive Films (Applied Biosystems)
- MicroAmp™ Optical 8-Cap Strips (Applied Biosystems)

1.5 The optimized singleplex real-time PCR detection protocols (setup: see Table 5) for GGI and GGII NVs were tested on 2 thermocyclers:

- ABI SDS7000 (Applied Biosystems)
- Lightcycler LC480 (Roche)

Table 5: Setup optimized real-time PCR reaction mix.

Component (GGI/GGII)	Final concentration
TaqMan uMMX	1x
Forward primer (QNIF4/QNIF2)	500 nM (GGI) / 500 nM (GGII)
Reverse primer (NV1LCR/COG2R)	900 nM (GGI) / 900 nM (GGII)
TaqMan probe (NVGG1p/QNIFS)	100 nM (GGI) / 250 nM (GGII)
Standard (pGI / pGII)	10-10^7 copies

2. Evaluation singleplex real-time PCR assay for detection of MNV-1.

The real-time PCR protocol for the detection of MNV-1 designed by Baert et al., 2008 (2) (Partner 2) was evaluated on 2 thermocyclers:

- ABI SDS7000 (Applied Biosystems)
- Lightcycler LC480 (Roche)

Setup:

Table 6: Setup singleplex MNV-1 real-time PCR detection assay

Product	Sequence (5' – 3')	Final conc.	Position in genome*
TaqMan uMMX (Applied Biosystems)		1x	
Forward primer (FW-ORF1/ORF2)	CACGCCACCGATCTGTTCTG	200 nM	4972-4991
Reverse primer (RV-ORF1/ORF2)	GCGCTGCGCCATCACTC	200 nM	5064-5080
MGB probe (MGB-ORF1/ORF2)	NED-CGCTTTGGAACAATG-MGBNFQ**	200 nM	5001-5015
Standard (p20.3)	Plasmid with full-size genome of the MNV-1.CW1 strain.	$10\text{-}10^7$ copies/reaction	

*Position in DQ285629-ref genome

** MGBNFQ: Minor groove binding nonfluorescent quencher

(2) Baert, L., Wobus, C. E., Van Coillie, E., Thackray, L. B., Debevere, J. & Uyttendaele, M. (2008). Detection of murine norovirus 1 by using plaque assay, transfection assay, and real-time reverse transcription-PCR before and after heat exposure. *Applied and Environmental Microbiology*, 74, 543-546.

B. Development of a multiplex real-time PCR assay for detection of GGI and GGII NVs and MNV-1.

All development and optimization steps of the multiplex assays were performed on the Lightcycler LC480 real-time PCR system under the following conditions: incubation at 50°C for 2 min to activate UNG, initial denaturation at 95°C for 10 min, followed by 50 cycles of amplification with denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Amplification data were collected and analysed with the instruments' software.

1. Optimization of GGI/GGII/MNV-1 multiplex assay on Lightcycler LC480 real-time PCR system.

1.1 Adjustment TaqMan probe fluorophore choice.

Since the Yakima Yellow (GGII) dye and the NED-dye (MNV-1) are detected in the same channel (Hex) of the LC480, the Yakima Yellow dye was replaced by the Texas Red dye.

1.2 GI/GII/MNV-1 multiplex.

The selected primers-probe sets for singleplex detection of GGI and GGII NVs were combined with the primers-probe set for the detection of MNV-1 in a multiplex real-time PCR assay. TaqMan uMMX was used as PCR mastermix and an optical Adhesive Film was used as seal type. Equimolar amounts of the pGI, pGII and p20.3 plasmids were used as 10-fold diluted standard series.

1.3 Examination of the competitive effects between the individual PCR reactions.

To examine possible competitive effects between the 3 individual assays within the multiplex assay, the effect of differential DNA concentrations on the outcome of the multiplex real-time PCR for each target was studied. To study this competition, all possible combinations of quantities of 0, 10, 10^3 and 10^5 copies of pGI, pGII and p20.3 were prepared (see table 7) and all combinations were tested in the multiplex PCR assay.

C. Analysis of the sensitivity and specificity of the multiplex real-time RT-PCR.

1. Specificity/Sensitivity analysis.

In order to evaluate the specificity and sensitivity of the multiplex assay, 15 clinical previously genotyped by the Belgian Scientific Institute of Public Health and the Rega Institute for Medical Research, a Norovirus Reference panel friendly provided by the National Institute for Public Health and the Environment (RIVM – The Netherlands) containing RNA transcripts from the ABC region of 9 GGI, 8 GGII and 1 GGIV noroviruses and finally 7 alternative virus strains (Astrovirus type 1&4, Rotavirus,

Sapovirus (SV), Feline Calicivirus (FCV), Canine Calicivirus (CCV) and Hepatitis A virus (HAV)) were subjected to our multiplex real-time RT-PCR assay. 2 negative samples and 1 unknown sample were also included.
RNA was extracted from clinical samples using the RNeasy Minikit (Qiagen). cDNA was prepared using the Multiscribe RT-kit (Applied Biosystems) in combination with random hexamers. One µl of the synthesized cDNA served as template in the real-time PCR assay in which TaqMan uMMX was used as PCR mastermix and an optical adhesive film was used as seal type. Equimolar amounts of the pGI, pGII and p20.3 plasmids were used as 10-fold diluted standard series.

2. Analysis of PCR inhibition.
Since inhibition is a frequently observed problem when detecting microorganisms in clinical samples or food matrices, inhibition controls in all steps of the detection protocol are a necessity. To avoid false-negative results due to PCR inhibition, 10^3 copies of the p20.3 plasmid were spiked in the real-time PCR reaction mix of all clinical samples as internal control. Obtained Ct values were compared to expected Ct values.

Validation of the CEN WG6 TAG4 real time PCR method to detect NVs in seafood/shellfish (P5).

1. Detection of NV in bivalves molluscs using the CEN WG6 TAG4 method

One gram of hepatopancreas was dissected from the shellfish. To obtain this material, 3 to 5 mussels or 1 to 2 oysters are necessary. The digestive glands were treated with protease K and RNA was extracted by the commercial kit Nucleospin®RNA virus (Macherey-Nagel) according to manufacturer's instructions. The RNA of the process control, mengovirus was spiked into the digestive glands of the molluscs and extracted at the same time than RNA of the samples. This control gives an indication on the extraction procedure, the inhibitory effects of the PCR reaction and the RT-PCR reaction it-self. A 1 step real time RT-PCR (Platinium® Quantitative RT-PCR Thermoscript™ One-Step System (Invitrogen™) was chosen in order to avoid contaminations in between the RT and PCR reactions. The internal RNA control (T+) recommended by the CEN protocol was quickly left out of the PCR plate as its use caused a major problem of contamination in our laboratory. Moreover, it took some time to eliminate the target sequence from the environment. A new T+ with a different target sequence was developed and is presented in the 6.2 section of this report. In each PCR plate was added a negative extraction control for GGI and GGII, a NTC (no template control) for each genogroup and 3 mengovirus controls (purified RNA, an extraction control and a NTC) for GGI and GGII.

Table7. The CEN WG6 TAG4 method primers and probes used in the real-time RT-PCR reaction.

Genogroup	Name	Sequence 5' - 3'	Amplicon size	Temperature	Reference
GGI	QNIF4	CGCTGGATGCGNTTCCAT			da Silva et al, 2007
	NV1LCR	CCTTAGACGCCATCATCATTTAC	85 bp	60°C	Svraka et al, 2007
	NV1LCpr	FAM-TGGACAGGAGAYCGCRATCT-TAMRA			Svraka et al, 2007
GGII	QNIF2	ATGTTCAGRTGGATGAGRTTCTCWGA			Loisy et al, 2005
	COG2R	TCGACGCCATCTTCATTCACA	89 bp	60°C	Kageyama et al, 2003
	QNIFS	FAM-AGCACGTGGGAGGGCGATCG-TAMRA			Loisy et al, 2005

DA SILVA, A. K., LE SAUX, J. C., PARNAUDEAU, S., POMMEPUY, M., ELIMELECH, M. & LE GUYADER, F. S. (2007) Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse transcription-PCR: different behaviors of genogroups I and II. Appl Environ Microbiol 73, 7891-7897
KAGEYAMA, T., KOJIMA, S., SHINOHARA, M., UCHIDA, K., FUKUSHI, S., HOSHINO, F. B., TAKEDA, N. & KATAYAMA, K. (2003) Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. J Clin Microbiol 41, 1548-1557
LOISY, F., ATMAR, R. L., GUILLON, P., CANN, P. L., POMMEPUY, M. & GUYADER, F. S. L. (2005) Real-time RT-PCR for norovirus screening in shellfish. Journal of Virological Methods 123

SVRAKA, S., DUIZER, E., VENNEMA, H., DE BRUIN, E., VAN DER VEER, B., DORRESTEIJN, B. & KOOPMANS, M. (2007) Etiological role of viruses in outbreaks of acute gastroenteritis in The Netherlands from 1994 through 2005. J Clin Microbiol 45, 1389-1394

A hundred and twenty-four samples (78 mussels, 42 oysters, 3 bittersweets and 1 shrimp) provided by the FASFC were analyzed for GGI and GGII noroviruses.

As National Reference Laboratory, we were able to participate to a couple of ring-tests. In the first one, organized by the Centre for Environment, Fisheries & Aquaculture Science (CEFAS, Weymouth), and all positive samples could be detected with our real time PCR method. In the second ring-test was organised by the CEFAS for the CEN WG.

2. Elaboration of a new internal RNA control (T+)

In order to solve the huge contamination problem we encountered at the start of the project using the internal control proposed by the CEN, a novel internal control (IC) was constructed. The new IC should not contain the target region of the real time PCR probes. A bacterial DNA sequence was chosen on which the GGI or GGII primer sequences were added at both ends. Indeed a new probe was designed to recognize the DNA target. To distinguish the amplification curves of the IC with those of the mengovirus controls and positive samples, a VIC probe has been constructed.

3. Determination of the detection limit of the real time RT-PCR for the detection of GGI NVs

Detection limits of both real time RT-PCR methods for GGI and GGII have been determined before the start of the project. Surprisingly, the detection limit of GGI was rather poor (over a thousand particles) and had to be determined again. A plasmid containing the CEN internal control sequence for GGI was sequenced and then linearized by digestion with SalI restriction enzyme (BioLabs). Synthetic RNA corresponding to the insert sequence was obtained by reverse transcription using the Riboprobe® kit (Promega) followed by a RQ1 DNase RNase-free treatment (Promega). RNA was purified (RNeasy® mini kit (Qiagen)) and its concentration (ng/µl) was determined by spectrometry (nanodrop). To make sure that the RNA sample is exempt of plasmid DNA, the sample was amplified by conventional PCR. Ten-fold dilutions were realized (3 repetitions for each dilution) before real time RT-PCR (40 cycles). Dilutions tested began at 10^{-3} up to 10^{-12}. The detection limit quantification was based on the lowest dilution detected and involving the molecular weight of 1 synthetic RNA copy (67267.6g/mole).

Optimization and validation of real-time RT-PCR methods for the detection of animal NV strains (C1).

1. Elaboration of a multiple species stool bank

Animal stool samples have been collected and sent to our laboratory through collaborations with various institutions. Stool samples from cattle were provided by ARSIA (Association Régionale de Santé et d'Identification Animales), porcine faeces were collected by Dierengezondheidszorg (DGZ) and equine faeces were provided by INTA (Instituto Nacional de Tecnología Agropecuaria) from Argentina. Stool samples from other animal species were provided by colleagues of the institution ULg (Poultry: Dr Marlier, domestic carnivores: Dr Zicola, equine: Dr Amory and murine: Dr Kesteloot and Dr Delforge). Samples were taken from animals with signs of gastro-enteritis with the exception of the murine species.

2. Screening of animal faecal samples by classical RT-PCR

In order to develop a real time RT-PCR for animal samples, we first have to select the species will be focused on. We selected several primer pairs that have been previously described as broadly reactive for caliciviruses and more particularly noroviruses in animal species: JV12(Y) - JV13 (I) and p290(d)-289(d). Moreover, for the bovine, caprine and ovine samples more specific primer pairs have been selected: CBECu F-R and BEC F-R. CBECU F-R was also used for the screening of the equine samples as no calicivirus has been described for this species yet. A novel primer pair swNoF-R has been designed for the specific detection of PoNoV. Sequences and detailed information of these primer pairs are shown in the table 8.

Table 8. Primer pairs used for the detection of Noroviruses in animal stool samples by conventional RT-PCR

Primer	Sequence 5' to 3'	Sense	Amplicon (bp)	Amplified region	Reference
JV12	ATACCACTATGATGCAGATTA	+	326	Polymerase region	Vinjé and Koopmans, 1996
JV13	TCATCATCACCATAGAAAGAG	-			
P290	ATACCACTATGATGCAGATTA	+	318		Jiang et al., 1999
P289	TGACAATGTAATCATCACCATA	-			
CBECU-F	AGTTAYTTTTCCTTYTAYGGBGA	+	532	ORF1/ORF2 junction	Smiley et al., 2003
CBECU-R	AGTGTCTCTGTCAGTCATCTTCAT	-			
BEC-F	GGGACCTTGARTTTGACCC	+	263	Polymerase region	Ike et al., 2007
BEC-R	GGTTGCTGTGGGGGACCA	-			
swNo F	AGGCAGCTCTATTGGACTAG	+	355	Polymerase region	Mauroy et al, 2008
swNo R	GGTCTCATTATTGACCTCTGG	-			

IKE, A. C., ROTH, B. N., BOHM, R., PFITZNER, A. J. & MARSCHANG, R. E. (2007) Identification of bovine enteric Caliciviruses (BEC) from cattle in Baden-Wurttemberg. Deutsche Tierarztliche Wochenschrift 114, 12-15
JIANG, X., HUANG, P. W., ZHONG, W. M., FARKAS, T., CUBITT, D. W. & MATSON, D. O. (1999) Design and evaluation of a primer pair that detects both Norwalk- and Sapporo-like caliciviruses by RT-PCR. Journal of Virological Methods 83, 145-154
MAUROY, A., SCIPIONI, A., MATHIJS, E., MIRY, C., ZIANT, D., THYS, C. & THIRY, E. (2008) Noroviruses and sapoviruses in pigs in Belgium. Arch Virol 153, 1927-1931
SMILEY, J. R., HOET, A. E., TRAVEN, M., TSUNEMITSU, H. & SAIF, L. J. (2003) Reverse transcription-PCR assays for detection of bovine enteric caliciviruses (BEC) and analysis of the genetic relationships among BEC and human caliciviruses. J Clin Microbiol 41, 3089-3099
VINJE, J. & KOOPMANS, M. P. (1996) Molecular detection and epidemiology of small round-structured viruses in outbreaks of gastroenteritis in the Netherlands. J Infect Dis 174, 610-615

Stool samples were diluted in 10% PBS and agitated for several hours at 4°C. After centrifugation, supernatants are collected and used for the RNA extraction. RNA was extracted using the commercial QIAamp® Viral RNA mini kit (Qiagen®). Each sample was first tested with degenerated JV12-JV13 primer pair with an internal control (IC) especially developed for this primer pair (Scipioni et al. Mol Cell Probes. 2008 Aug;22(4):215-22). The IC enables the visualization of inhibitory effects during RT-PCR. If inhibition was observed in a sample, it could be eliminated by diluting the RNA by ten-fold. The use of Bovine Serum Albumin (BSA) at a concentration of 200 to 400ng/µl could also relief amplification inhibition without having to dilute the RNA. RT and PCR were performed in a one-step procedure using the commercial Access® RT-PCR system (Promega®) following manufacturer's recommendations. RT-PCR products were analyzed on 2% agarose gel electrophoresis and stained with ethidium bromide. The RT-PCR product bands were visualized by using UV light. Samples were considered as positive when RT-PCR products were observed at the expected amplicon size (see tables above).

Work package 2: Virus evolution

Genotyping and study of recombinant viruses, will be started with the detection of NVs strains, and will be carried on in years 3 and 4 (C1 and P5, with input of P3).

1. Sequencing of the positive results obtained by classical RT-PCR and Sybergreen real-time RT-PCR

Human positive stools collected from IPH and UCL, were confirmed positive by primer pair JV12/13 for the polymerase region. Also isolated cases of gastro-enteritis with suspicion of NV infection were analysed. Other more specific primer pairs were used for the amplification of different regions in the genome of yet confirmed positive stool samples. Characteristics of these primer pairs are given in table 9 and all primer pairs used for the genotyping are presented in the figure 1 (see below).

Table 9. Primer pair sets used for genotyping of noroviruses.

Primer	Sequence 5' to 3'	Sense	Amplicon (bp)	Amplified region	Reference
GISKF	CTGCCCGAATTYGTAAATGA	+	330	Capsid region	Kojima et al, 2002
GISKR	CCAACCCARCCATTRTACA	-			
GIISKF	CNTGGGAGGGCGATCGCAA	+	344		
GIISKR	CCRCCNGCATRHCCRTTRTACAT	-			
1531	GCACTCGGCATCATGACAAAATTCA	+	1.481	Polymerase region	La Rosa et al, 2008
1565	GGAACTGAACAACTTGGGGT	-			
GII.4 NS1	AACGACACCGCAAAATCTTC	+	1.494	NS1-helicase	In this study
GII.4 NS3	GGAGGCTGCGATTCTCTTAG	-			

KOJIMA, S., KAGEYAMA, T., FUKUSHI, S., HOSHINO, F. B., SHINOHARA, M., UCHIDA, K., NATORI, K., TAKEDA, N. & KATAYAMA, K. (2002) Genogroup-specific PCR primers for detection of Norwalk-like viruses. Journal of Virological Methods 100
LA ROSA, G., POURSHABAN, M., IACONELLI, M. & MUSCILLO, M. (2008) Detection of genogroup IV noroviruses in environmental and clinical samples and partial sequencing through rapid amplification of cDNA ends. Arch Virol 153, 2077-2083

The expected size amplicons were excised from the agarose gel for DNA purification. The commercial kit, QIAquick® Gel Extraction Kit (Qiagen®), was used for this purpose according to the manufacture's recommendations. The purified DNA was either directly sent for sequencing to the GIGA Genomics Facility or cloned into a pGEM®-T Easy vector (Promega®) before sequencing.

The sequences were blasted on the NCBI website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and aligned with clustal W with published sequences available in Genbank. Sequences from the polymerase region and the capsid region were furthermore submitted to a quicktyping tool available to the public on the internet at https://hypocrates.rivm.nl/. A phylogenetic analysis was conducted in MEGA4 with the construction of trees using the Neighbor-Joining method and distances between sequences were computed using the Maximum Composite Likelihood method.

2. Production of long DNA fragments from positive stool samples

For genotyping and the study of NV recombinants, it is essential to work with sequences that cover the ORF1/ORF2 junction including the complete capsid sequence (ORF2). Therefore, large fragments were amplified of nearly 3.5 kb. Fragments were amplified from the end of ORF1 through the polyadenylated tail of the genome. These transcripts were obtained by 2-step RT-PCR. The RT step was performed using a Linker primer that recognizes the polyA tail and the Superscript™ Reverse Transcriptase (Invitrogen™) procedure. cDNA was amplified by the iProof™ High-Fidelity DNA Polymerase (Bio-rad) using JV12Y/p290d/1531 and Linker as primers. The large RT-PCR products were analyzed on 0.8% agarose gel electrophoresis and stained with ethidium bromure. The RT-PCR product bands were visualized by using UV light. All product bands that showed a size of roughly 3.5 kb were excised and purified as described above. DNA was cloned into vectors designed for large fragments according to the manufacturer's instructions (Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen™, TOPO XL® PCR Cloning Kit Invitrogen™) before being sequenced by primer walking. When long fragments could not be realized, the ORF1-ORF2 junction was amplified by the primer pair JV12Y-GIISKR or JV12Y-GISKR generating an amplicon of approximately 1000 bp.

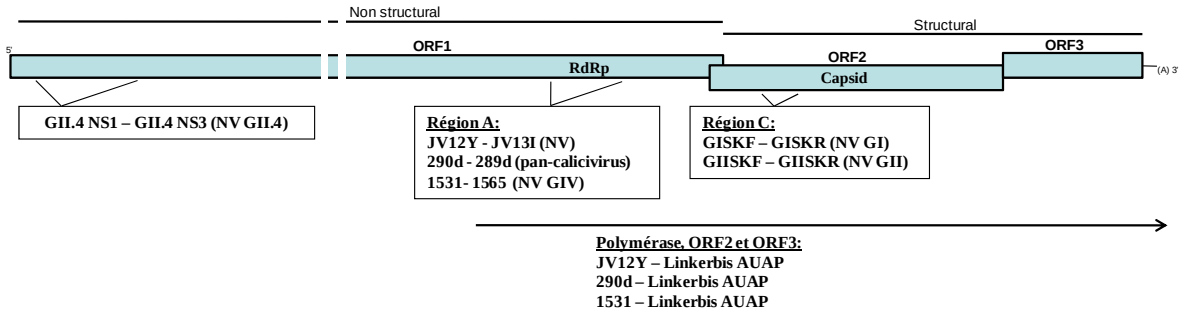

Figure 1. Representation of different primer pairs used for the genotyping and the study of recombinant viruses detected in positive human stool samples.

3. Sequencing of NV strains detected in shellfish

With the input of the C1 partner, sequencing of NV strains from positive samples was realized. Two different approaches have been tested: direct sequencing of the nested-PCR products (after gel excision and DNA purification), sequencing of RT-PCR products amplified with primer pairs JV12-13, p290-289 (amplifying in the polymerase region) and CNVI F/R-CNVII F/R (capsid region).

Work package 4: Development of a Network (P3)

Currently, in Belgium, there is no specific procedure to trace NV outbreaks and to establish the link between human epidemic and food contamination. Since the reform of Belgium into a Federal State with Regions and Communities there is a need for coordination between the different partners implicated in outbreak monitoring. Since food is a federal matter and person related matters such as illness are the competence of the Flemish, French and German communities, data on food-borne outbreaks are now very dispersed. Communication, exchange of information, a better data collection from outbreak investigations and case-control studies have yet been improved by the creation of a **National Platform for Diseases Transmitted by Food** in the Institute of Public Health. However, a field and laboratory scenario has to be worked out for a better coupling of NV outbreaks to their food-borne cause and to elucidate the transmission routes of NV strains circulating in human, animals and food.

IV. RESULTS

Work package 1: Methods of analysis

Optimization and validation of real-time RT-PCR methods for detection of human genotypes (GGI and GGII) of NVs and for MNV-1 (P4, with input of P2 and P3).

A. Optimization of singleplex real-time PCR assays for detection of GGI and GGII NVs and for MNV-1

1. Optimization singleplex real-time PCR assays for detection of GGI & GGII NVs.

1.1 Comparison of different PCR mastermixes

This comparison made clear that the use of commercial mastermixes (MMXs) resulted in a higher reproducibility, a lower detection limit and a higher PCR-efficiency. The results obtained by the use of the GeneExpression MMX and the TaqMan uMMX were comparable, but remarkably better than the results of the BlueRox MMX. Eventually the TaqMan uMMX was selected for further use.

1.2. Comparison of different primers-probe sets

A comparison of parameters of the standard curves of duplicates of 2 independent runs in which both primers-probe sets were used showed that both primers-probe sets resulted in comparable R^2-values and sensitivity (see Table 10 & Table 11).

Table 10: Standard curve parameters when primers-probe set designed by Jothikumar et al., 2005 was used.

Genogroup	Det. Limit (copies/reaction)	PCR-efficiency	R^2-value	Ct 10^7 copies
GGI	10	121.83 %	0.99	23
GGII	100	113.28 %	0.97	23

Table 11: Standard curve parameters when primers-probe set designed by CEN/TC/WG6/TAG4 was used.

Genogroup	Det. Limit (copies/reaction)	PCR-efficiency	R^2-value	Ct 10^7 copies
GGI	10	112.75 %	0.99	19
GGII	100	113.81 %	0.96	21

The CEN/TC/WG6/TAG4 primers-probe set was chosen for further use to detect GGI & GGII NVs by real-time RT-PCR.

1.3. Comparison of different types of template as real-time PCR standard (positive controls).

The use of plasmids (pGI and pGII) as real-time PCR standard was considered after the occurrence of a great number of positive NTCs (No Template Controls) when using synthetic single stranded DNA-fragments.
Table 12 shows that there are no unexpected differences between the results obtained when plasmids or synthetic fragments are used. A 1 Ct difference between the ssDNA and plasmid standard curves was expected, since the ssDNA fragments require an extra amplification cycle to become double-stranded target DNA.
However, the frequency of positive NTCs was seriously reduced when plasmids were used as standard. For this reason, plasmids were chosen for further use as positive control in a 10-fold diluted standard series (10^7 – 10 copies).

Table 12: Comparison of ssDNA fragments and pGI/pGII as real-time PCR standard.

Genogroup	Template	Det. Limit (copies/reaction)	PCR-efficiency	R^2-value	Ct 10^5 copies
GGI	Fragment	10	112,75%	0.99	25.84
	pGI	10	107,71%	0.99	24.62
GGII	Fragment	10	104,89%	0.99	27.45
	pGII	10	98,43%	0.99	24.89

1.4 Two different sealing systems of the 96-well real-time PCR reaction plates were compared:

The use of the optical adhesive films was preferred over the use of the optical cap strips, since the latter gave cause to a mild disturbance of the fluorescence signal.
This disturbance resulted in a dramatic reduction in the reproducibility (R^2-value = 0.834). The use of the adhesive film resulted a higher R^2-value = 0.997

1.5 The optimized singleplex real-time PCR detection protocols for GGI and GGII NVs were tested on 2 different thermocyclers

Table 13 shows that acceptable results were obtained on the 2 tested thermocyclers.

Table 13: Evaluation of optimized singleplex real-time PCR GGI and GGII assays on 2 thermocyclers

Thermo-Cycler	Genogroup	Det. limit (copies/reaction)	PCR-efficiency	R^2-value	Ct 10^5 copies
SDS7000	GGI	10	107,71%	0.99	24.62
	GGII*	10	98,43 %	0.99	24.89
LC480	GGI	10	90.94 %	1.00	23.93
	GGII*	10	95.68 %	0.99	25.28

* Yakima Yellow labeled TaqMan probe

2. Evaluation singleplex real-time PCR assay for detection of MNV-1.

These results (Table 14) show that this real-time PCR assay is appropriate for the real-time PCR detection of MNV-1.

Table 14: Evaluation singleplex real-time PCR assay for detection of MNV-1.

Thermo-Cycler	Det. Limit (copies/reaction)	PCR-efficiency	R^2-value	Ct 10^5 copies
SDS7000	10	94.92 %	1.00	23.96
LC480	10	94.17 %	1.00	22.33

B. Development of a multiplex real-time PCR assays for detection of GGI and GGII NVs and for MNV-1.

1. Optimization of GGI/GGII/MNV-1 multiplex assay on Lightcycler LC480 real-time PCR system.

1.1 Adjustment TaqMan fluorophore choice.

Table 15 shows that a slightly reduced PCR-efficiency is noticed when Texas Red is used as TaqMan dye instead of Yakima Yellow. This reduced PCR-efficiency remains sufficient for a correct quantitative real-time PCR assay. Moreover, this reduction was not noticed when all singleplex assays were combined into the multiplex assay (see Table 16).

Table 15: Comparison of standard curve parameters when Yakima Yellow or Texas Red was used as TasMan dye.

Genogroup	Fluorophore (5')	Det. limit (copies/reaction)	PCR-efficiency	R^2-value	Ct 10^5 copies
GGII	Yakima Yellow	10	95.68 %	0.99	25.28
GGII	Texas Red	10	88.25 %	1.00	23.98

1.2 GI/GII/MNV-1 multiplex.

A comparison of parameters of the standard curves of duplicates of 5 independent multiplex runs with those of 2 independent singleplex runs showed that Cts are in accordance with each other, with a maximum difference of less than 1.
The individual GI/GII/MNV-1 assays within the multiplex PCR are sensitive, efficient and reproducible. These data suggest that reliable detection of the GI/GII NVs and MNV-1 within the same sample is possible on the LC480 instrument using the triplex real-time PCR assay.

Table 16: Comparison individual GI/GII/MNV-1 assays and multiplex assay

Genogroup	PCR format	Det. Limit (copies/reaction)	PCR-efficiency	R^2-value	Ct 10^5 copies
GGI	singleplex	10	90.94 %	1.00	23.93
GGI	multiplex	10	98.84 %	1.00	23.47
GGII*	singleplex	10	88.25 %	1.00	23.98
GGII*	multiplex	10	93.43 %	1.00	23.92
MNV-1	singleplex	10	94.92 %	1.00	21.21
MNV-1	multiplex	10	95.30 %	1.00	20.66

* Texas Red labeled TaqMan probe

1.3 Examination of the competitive effects between the individual PCR reactions.

An overview of the results is shown in figure 2.

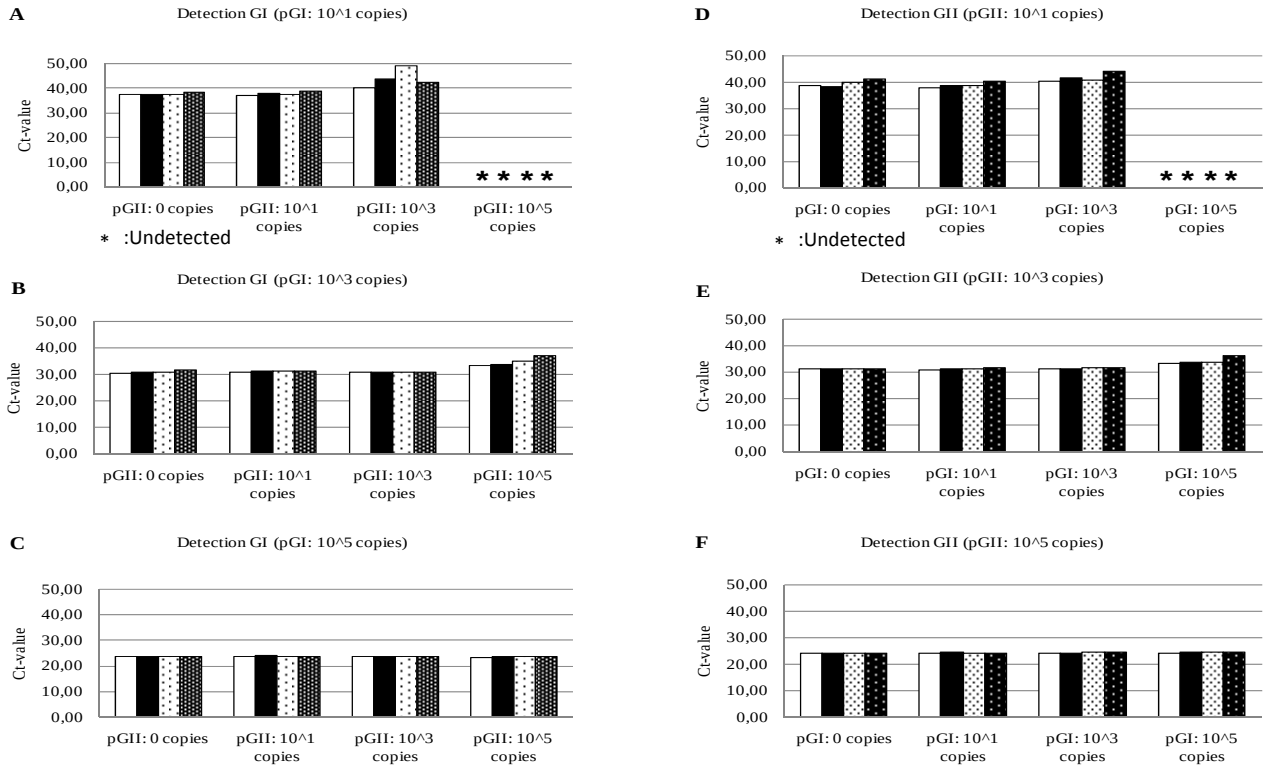

Figure 2. A-B-C: The effect of the presence of GII on Ct values (vertical axis) of the GI reaction within the multiplex real-time PCR assay. Different copy numbers (0, 10, 10^3 and 10^5 copies) of pGII (horizontal axis) are combined with 10 (fig 2A), 10^3 (fig 2B) and 10^5 (fig 2C) copies of pGI. **D-E-F**: The effect of the presence of GI on Ct values (vertical axis) of the GII reaction within the multiplex real-time PCR assay. Different copy numbers (0, 10, 10^3 and 10^5 copies) of pGI (horizontal axis) are combined with 10 (fig 2D), 10^3 (fig 2E) and 10^5 (fig 2F) copies of pGII. The effect of the presence of MNV-1 on the GI and GII reactions within the multiplex real-time PCR assay was also included in this figure 1. Quantities of 0 (series □) 10 (series ■), 10^3 (series ⊡) and 10^5 (series ▩) copies of p20.3 were combined with any combination of copy numbers of pGI and pGII. All Ct values are means of duplicates.

The effect of the presence of GGII on the GGI reaction within the multiplex assay was not negligible (fig 2 A-B-C). 10^5 copies of plasmid pGI – containing primers-probe binding sites of GGI NVs – were detected at the expected Ct value in the presence of 10, 10^3 and 10^5 copies of pGII – containing primers-probe binding sites of GGII NVs – (fig 2A). 10^3 copies of pGI were detected at the expected Ct value in the presence of 10 or 10^3 copies of pGII, while a 2.8 to 6.6 Ct increase was noticed in the presence of 10^5 copies of pGII (fig 2B). Ten copies of pGI were detected at the expected Ct value in the presence of 10 copies of pGII. However, a 2.8 to 11.7 Ct-shift was noticeable in the presence of 10^3 copies of pGII while ten copies of pGI could not be detected (Ct>50) in the presence of 10^5 copies of pGII (fig 2C).

Similarly, the presence of GGI affected the GGII reaction within the multiplex assay when high amounts (10^5 and 10^3 copies) of pGII were combined with any copy number (0, 10, 10^3 and 10^5 copies) of pGI (fig 2D, 2E). An alike 2.2 to 5.0 Ct-shift was noticeable when 10^3 copies of pGI were detected in the presence of 10^5 copies of pGII (fig 2E). Ten copies of pGII were detected as expected in the presence of 10 copies of pGI. However, a 1.8 to 5.6 Ct-shift was noticeable in the presence of 10^3 copies of pGI while ten copies of pGII could not detected (Ct>50) in the presence of 10^5 copies of pGI (fig 2F).

Overall, the effect of the MNV-1 reaction on the GGI and GGII reactions within the multiplex assay was limited when pGI or pGII were solitarily present, as only a 4 log excess (10^5 copies) of plasmid p20.3 (containing a MNV-1 genome insert) over pGI and/or pGII (10 copies) caused a Ct-shift ranging from 1 to 3 Cts (fig 1A and 1D). On the other hand, the effect of the MNV-1 reaction on the GGI and GGII reactions within the multiplex assay was not negligible when pGI and pGII were both present. When 10 and 10^3 copies of pGI were combined with respectively 10^3 and 10^5 copies of pGII, Ct-shifts respectively ranging from 2.11 to 8.91 and 1.56 to 3.84 were caused by the presence of 10^3 or 10^5 copies of p20.3. Similarly, when 10 and 10^3 copies of pGII were combined with respectively 10^3 and 10^5 copies of pGI, Ct-shifts respectively ranging from 0.48 to 3.83 and 0.23 to 3.83 were caused by the presence of 10^3 or 10^5 copies of p20.3.

C. Application of the multiplex real-time RT-PCR for detection of GGI/GGII NVs and MNV-1.

1. Specificity/Sensitivity analysis.

The results summarized in table 17 show that all tested genotypes present in the Norovirus RNA reference panel were detected and no cross-amplification between the different GGI and GGII genotypes occurred. The Alphatron genotype (GGIV) was detected with the GGI assay.

All clinical samples previously found positive for GGI (5 samples) or GGII (10 samples) NVs were also found positive in the multiplex assay and again no cross-amplification occurred between the GGI and GGII genotypes (see table 18). No amplification occurred in the negative samples, whereas the unknown sample turned out to be a GGII NV sample.

All other alternative viruses were found negative in the multiplex real-time RT-PCR (see Table 19).

Table 17: Overview of different genotypes present in Norovirus RNA Reference Panel.

Genotype	Ct GGI	Ct GGII	Ct MNV-1
GI.1 (Norwalk)	29.01	Undet	Undet
GI.2 (Whiterose)	20.52	Undet	Undet
GI.2 (Southampton)	20.83	Undet	Undet
GI.3 (Birmingham)	19.09	Undet	Undet
GI.4 (Malta)	19.33	Undet	Undet
GI.5 (Musgrove)	39.12	Undet	Undet
GI.6 (Mikkeli)	19.62	Undet	Undet
GI.7 (Winchester)	17.54	Undet	Undet
GI.10 (Boxer)	19.34	Undet	Undet
GII.1 (Hawaii)	Undet	19.46	Undet
GII.2 (Melksham)	Undet	18.66	Undet
GII.3 (Toronto)	Undet	21.78	Undet
GII.4 (Grimsby)	Undet	18.26	Undet
GII.6 (Seacroft)	Undet	22.07	Undet
GII.10 (Erfurt)	Undet	18.49	Undet
GIIb (GGIIb)	Undet	19.05	Undet
GIIc (GGIIc)	Undet	19.21	Undet
GIV (Alphatron)	35.87	undet	Undet

Table 18: Overview of tested clinical samples.

Name sample	Genotype	Ct GGI	Ct GGII	Ct MNV-1
Negative		Undet	Undet	28.04
Negative		Undet	Undet	27.85
Unknown	GII.?	Undet	21.66	28.81
LVR 1	GI.2 (2003)	29.79	Undet	27.70
LVR 2	GI.2 (2004)	28.07	Undet	27.69
LVR 3	GI.4 (2006)	26.04	Undet	27.56
LVR 4	GI.8 (2007)	22.76	Undet	27.32
LVR 5	GII.2 (2007)	Undet	29.92	27.76
LVR 6	GII.4 (2002)	Undet	28.95	27.83
LVR 7	GII.4 (2002)	Undet	22.90	27.82
LVR 8	GII.4 (2007)	Undet	21.63	28.78
WIV 101	GII.?	Undet	28.92	27.79
WIV 193	GII.?	Undet	26.30	27.41
WIV 206	GII.?	Undet	33.57	27.89
WIV 242	GII.?	Undet	25.72	27.37
WIV 244	GII.?	Undet	26.28	27.61
WIV 246	GI.?	38.46	Undet	27.89
WIV 248	GII.?	Undet	27.05	27.58

Table 19: Overview of alternative viruses tested

Virus type	Ct GI	Ct GII	Ct MNV-1
Sapovirus	Undet	Undet	Undet
Rotavirus	Undet	Undet	Undet
Astrovirus 1	Undet	Undet	Undet
Astrovirus 4	Undet	Undet	Undet
FCV	Undet	Undet	Undet
CCV	Undet	Undet	Undet
HAV	Undet	Undet	Undet

2. Analysis of PCR inhibition.

10^3 copies of the p20.3 plasmid added to the cDNA preparation of all clinical samples were detected at the expected Ct – value (~28), suggesting that no PCR inhibitory components are present in the cDNAs of the clinical samples (see Table 18, right column).

Validation of the CEN WG6 TAG4 real time PCR method to detect NVs in seafood/shellfish (P5).

1. Detection of NV in bivalves mollusks using the CEN WG6 TAG4 method

The results are given in the table 20.

Table 20. Results of norovirus detection in mollusks Mai 2007 until December 2008

Matrices	Number of samples analyzed	Positive samples for NV	GG I	GG II	GG I+II
Mussels	238	35	9	32	6
Oysters	44	5	1	4	-
Bittersweets	3	1	-	1	-
Total	285	41	10	37	6

Fourty-one of the 285 analyzed samples (35 mussels, 5 oysters and 1 bittersweet) were found positive. Of these samples were positive 10 (24%) for GGI and 37 (90%) for GGII respectively. In 2 of the positive samples *Salmonella* was also detected, indicating shellfish contamination most probably occurred by faecal route. Unfortunately, samples were not systematically analyzed for the presence of *Salmonella*. Interesting was the simultaneous detection of both NV GGI and GGII in the same sample for six mussel matrices. The contamination of shellfish by different genogroups or strains at the same time could give birth to recombination events.

As the samples were analyzed by a semi-quantitative real-time RT-PCR, values of the Threshold cycle could give an indication of the quantity of viral RNA present in the sample. Ct values for positive samples were high (average of 38 Ct) probably indicating a low viral load in the shellfish. It would be better to dispose of shellfish that have been linked to an outbreak. It is difficult to determine whether the mollusks detected positive for NV are proper for human consumption or not. Volunteer studies have demonstrated that less than 10 viral particles of NV are capable of causing an infection after ingestion. On top of this, the question of the infectivity of the RNA detected by PCR is yet not answered and remains a critical point in the absence of an adequate cell culture enabling cultivation of NV.

The results from the 2 ring-tests were very satisfying. Conclusions of the second ring-test were the following:

- Sensitivity (detection of the lowest virus concentration) was similar for all laboratories for the detection of NVs GGII, Hepatitis A virus (HAV) and mengovirus. More variability of sensitivity was observed for GGII.
- **NVs GGI**: 5 laboratories on 17 detected until -5 of the provided sample. We detected at a -4 dilution, compromising within the 9 best results.
- **NVs GGII**: 14 laboratories on 16 detected dilutions between -4 and -6.
- **HAV**: 16 laboratories on 17 detected dilutions between -4 and -6.
- **Mengovirus** : 15 laboratories on 17 detected dilutions between -4 and -5.

For GGII, HAV and mengovirus, our laboratory obtained similar results to the majority of laboratoria.

A novel ring-test was organized by the CEFAS in December 2008. We were able to detect all positive matrices that were sent to us. These results are rather encouraging placing our laboratory within the best.

2. Elaboration of a new internal RNA control (T+)

The use of this IC has shown encouraging results until now. Our NTC do not show any signs of contamination since.

3. Determination of the detection limit of the real time RT-PCR for the detection of GGI NVs

The method detected the 3 samples diluted at 10^{-10} and only 1 samples diluted at 10^{-11}. The detection limit was 34.8 RNA particles when we took in account the 10^{-10} dilution. This result correlates with results for GGII (25-250 RNA particles detected). Note this is a preliminary result because the number of cycles should be extended upon 50 and the results will be affined by repeating dilutions in a more interesting range.

Optimization and validation of real-time RT-PCR methods for the detection of animal NV strains (C1).

1. Elaboration of a multiple species stool bank

Stool samples have been collected for the past two year and the stool bank is now composed of 799 faecal samples of different animal origins. The stool bank is composed as the followed:

Bovine : n= 480 (ARSIA, Dr Lomba and Maquet)
Ovine : n= 7 (ARSIA, Dr Lomba and Maquet)

Caprine : n= 2 (ARSIA, Dr Lomba and Maquet)
Equine : n= 101 (ULg, Dr Amory and INTA, Dr Barrandeguy)
Porcine : n= 43 (DGZ Torhout, Dr Miry)
Canine : n= 60 (ULg, Dr Zicola)
Feline : n= 36 (ULg, Dr Zicola)
Avian : n= 66 (ULg, Dr Marlier)
Murine : n= 3 (ULg, Mr Delforge, Dr Kesteloot)
Guinea pig : n= 1 (ARSIA, Dr Lomba and Maquet)

2. Screening of animal faecal samples by classical RT-PCR

The screening results are summarized in table 21.

Table 21. Results of the screening of the multiple species stool bank.

Species	Primer pair	Amplicons with expected size	Blast of amplicon sequence
Bovine	CBECu F/R	48	48 BoNoV GIII.2, GIII.1, Thirsk-like (GIII.1/GIII.2)
Equine	290d/289d	12	Aspecific amplification : Bacteria
Porcine	290/289	5	5 PoSaV
	swNo F/R	2	2 PoNoV GII.19
Feline	290d/289d	5	5 FCV
Canine	290d/289d	7	5 FCV 2 calicivirus polymerase-like sequences
Poultry	290d/289d	8	8 calicivirus polymerase-like sequences

NA: not applicable

The pan-calicivirus primer pair failed to detect bovine noroviruses and porcine noroviruses indicating that a single animal norovirus detection method will be difficult to set up. All ovine, caprine, equine, murine samples were negative. Twelve equine samples presented an amplification amplicon around the expected size but sequencing revealed an aspecific amplification of bacteria.

Positive samples of human stools were kindly provided by IPH (partner 3 of the network). These samples were already tested positive by real-time PCR and confirmed in our laboratory by Sybergreen with JV12Y-JV13I. Partial sequences of the capsid region were also amplified for the human samples for genotyping using the primer pair GIISKF/R mentioned above. Results of the genotyping are presented in the table below in part 2.1.

Work package 2: Virus evolution

Genotyping and study of recombinant viruses, will be started with the detection of NVs strains, and will be carried on in years 3 and 4 (C1and P5, with input of P3).

1. Sequencing of the positive results obtained by classical RT-PCR and Sybergreen real-time RT-PCR

Results for the animal stool samples are reported in table 21.

Most of the BoNoV sequences clustered with GIII.2 (prototype strain Newbury) and only few sequences clustered with GIII.1 (prototype strain Jena). This is rather unexpected as both bovine NV genotypes have been described in Europe (Germany, United Kingdom, and The Netherlands) as well as in the United States and South Korea. Some of the BoNoV clustered with the strain Thirsk, a previously described naturally recombinant strain. These BoNV showed high nucleotide and amino acid identities with the capsid gene of GIII.2, whereas the nucleotide and amino acid sequences of the RNA polymerase

gene were more closely related to those of GIII.1, suggesting that they belong to a GIII.1/GIII.2 recombinant strain. To exclude a co-infection, a long fragment was amplified covering part of the polymerase region to the polyadenylated end. When the sequence was submitted to Simplot analysis a putative recombination breakpoint was observed at the ORF1-ORF2 junction.

For the porcine species, NV strains were been detected and correlated with the fact that NV have been detected in pigs in the Netherlands. Both porcine NV strains belong to the GII.19 genotype. They were detected in young animals of about 16-20 weeks old. Interestingly, 5 porcine sapovirus (SV) sequences were found and correlates with finding in pigs from Hungary and Italy. The porcine sapovirus strains were genetically related to the porcine enteric calicivirus Cowden reference strain and to newly described porcine strains in the genus Sapovirus. These results confirm the presence of porcine NV and SV in Belgian pigs.

The sequencing results of dog and cat samples both indicated the presence of feline calicivirus showing FCV might cross the species barrier. Blasting of the sequences coming from canine stools with the canine norovirus recently isolated did not show any similarity.

Some amplicons obtained with primer pair 290d/289d in dogs and in poultry could not be aligned with any of the sequences part of the Genbank database when the nucleotide sequence was taken into account. When these sequences were translated into an amino acid sequence, the latter showed various conserved motifs of the calicivirus polymerase family more particularly those of the Sapovirus genus. These sequences might reflect the presence of a unknown calicivirus. Unfortunately, further investigations of the genome did not give any results yet.

Sequences obtained for human stool samples were obtained either by direct sequencing of the Sybergreen RT-PCR products or products from conventional RT-PCR (JV12Y-JV13I and GIISKF/R). Sequencing was successful in 27 of the 30 human fecal samples. In total, 17 of the 27 samples analyzed could be identified as being members of GGII genotype 4 (GII-4) know as the most prevalent NV strains circulating across the world. Co-infection by two different genotypes was observed in one case of isolated gastro-enteritis. In this sample both genotype II.4 2006b and genotype IV.1 was detected. Presently there were no potentially recombinant strains detected in this sample. For some samples it was not possible to obtain sequences for both the polymerase and the capsid region. Unfortunately these samples were totally explored and no initial material was left for further investigation. Results for human sample genotyping are presented in table 22. Results show predominant circulation in 2008 of GII.4 strains. In 2009, a more diverse pattern was observed. GII.4 strains were still circulating together with other GGII genotypes. In one outbreak, a GI.4 was detected and in one isolated gastro-enteritis case both GII.4 and GIV.1 was detected. Early 2008, a Sapovirus GI.2 strain was detected once in an isolated case originated from the North of France and no Belgian outbreak could be linked with the presence of a member of the Sapovirus genus.

Table 22. Results of detection and characterization by quicktyping of norovirus strains involved in gastro-enteritis outbreaks and isolated cases from 2004 to 2008 in Belgium.

Norovirus	Samples	Region A (polymerase)	Region C (capside)	ORF1-ORF2 junction	Polymerase, ORF2 and ORF 3
2004	UCL 5	GIIb	GII.3	GIIb-GII.3	-
2006	UCL 4	GII.4 2004	GII.4 2004	-	GII.4 2004
	UCL 6	GIIb	GII.3	-	-
2007	UCL 1	-	-	-	-
	UCL 2	GII.4 2006a	-	-	-
	UCL 3	GII.4 2006a	GII.4 2006a	-	-
	ISP 55	GII.4 2006b	-	-	-
	ISP 56	GII.4 2006b	-	-	-
	ISP 57	GII.4 2006b	GII.4 2006b	-	-
	ISP 58	-	-	-	-
	ISP 59	GII.4 2006b	-	-	-
	ISP 472	GII.4 2006a	GII.4 2006a	-	GII.4 2006a
	ISP 473	GII.4 2006a	GII.4 2006a	-	-
	ISP 474	GII.4 2006a	GII.4 2006a	-	GII.4 2006a
	ISP 475	GII.4 2006a	GII.4 2006a	-	GII.4 2006a
	ISP 476	-	-	-	-
	ISP 477	GII.4 2006a	GII.4 2006a	-	-
2008	Ange	GII.4 2006b GIV.1	GII.4 2006b -	GII.4 2006b -	- GIV.1
	Jeane	GII. ?	-	-	-
	ISP 333	GII.2	-	-	-
	ISP 335	GII.2	-	-	-
	ISP 336	GII.2	-	-	-
	ISP 356	GII.4 2004	-	-	-
	ISP 358	GII.4 2004	-	-	-
	ISP 360	GI.4	-	-	-
	ISP 363	GI.4	-	-	-
	ISP 374	GII.4 2006b	-	-	-
	ISP 375	GII.4 2004	-	-	-
	ISP 379	GII. ?	-	-	-
Sapovirus					
2008	Bene	GI.2	-	-	GI.2

Phylogenetic analyses were conducted both for the polymerase and the capsid partial regions and only two samples clustered differently according to the region (Figure 3.A and 3.B). For UCL6 and UCL5 potential recombinants were identified as the polymerase region clustered with GIIb strains whereas the capsid region clustered with GII.3 strains. To confirm UCL5 and UCL6 as recombinant it was essential to amplify the ORF1-ORF2 junction. For UCL5 it was possible to amplify this region by using the primer pair 290d/GIISKR. In order to confirm the recombination event, the obtained sequence (1.050 bp) was submitted to Simplot program analysis (version 3.5.1) comparing the similarity of the sequence of UCL5 with a GIIb (Pont de Roide: NV GIIb/GII.2) and a GII.3 (Toronto) reference sequence. The putative recombination breakpoint could be estimated by this analysis and was located close to the ORF1-ORF2 overlap region (Figure 4).

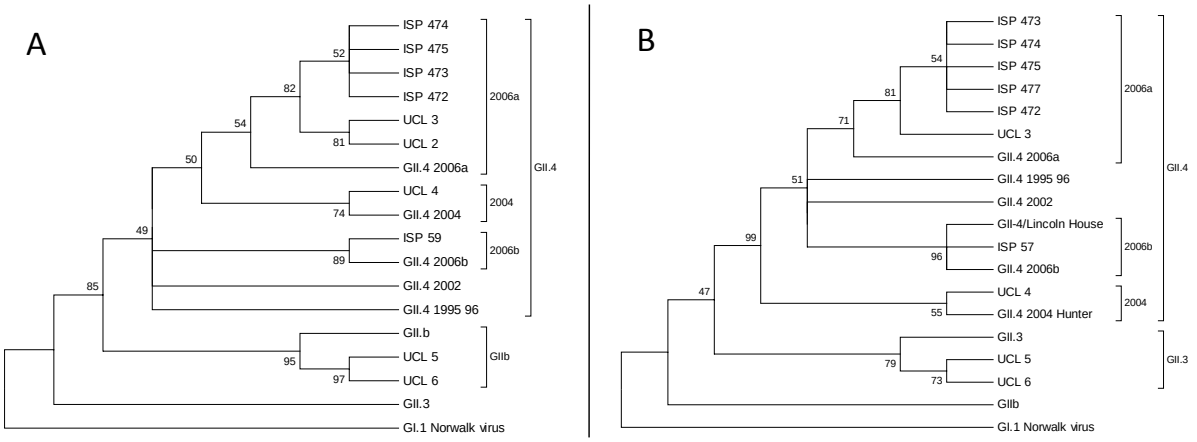

Figure 3.A. Tree based on partial sequences of the polymerase region of human NVs from positive human stools. B. Tree based on partial sequences of the capsid region of human NVs from positive human stools

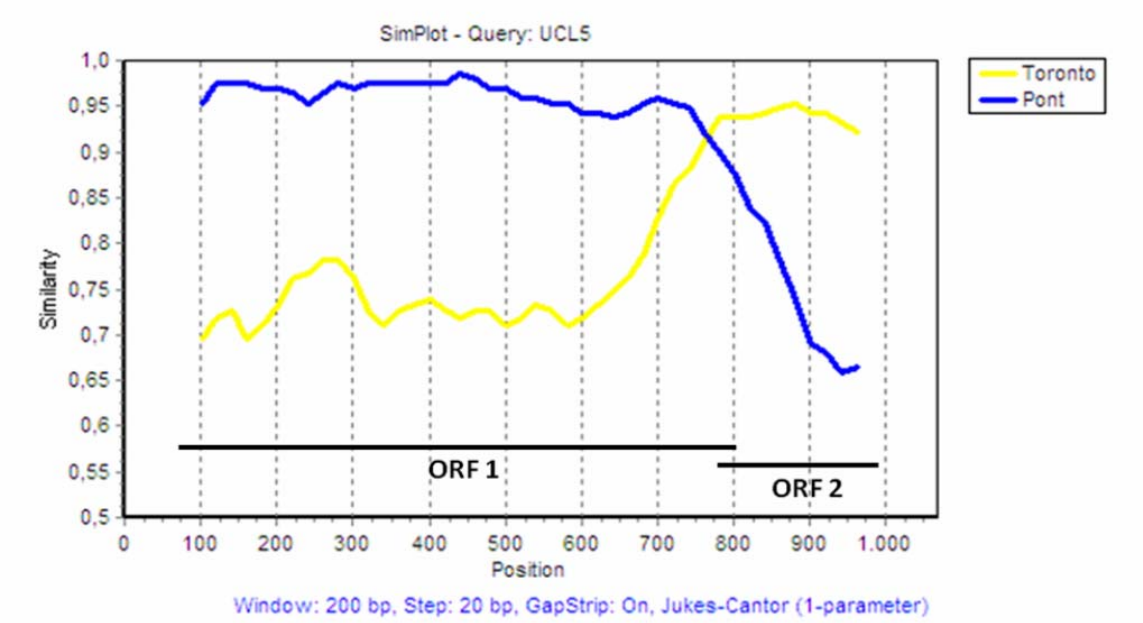

Figure 4. Similarity plot of UCL 5 sequence (1050 bp). Y-axis gives indicates the percentage of similarity of UCL 5 with the two putative parental strains Toronto (Accession number: U02030) and Pont de Roide (Accession number: AY682549). The site where the 2 parental strains have equal identity to the recombinant (i.e., where the lines cross) is the predicted site of recombination.

2. Production of long DNA fragments

To date only six fragments of approximately 3200 pair bases could be amplified. Three clones of each fragment were fully sequenced by primer walking and blast results are reported in the table above. A lot of attempts were necessary to obtain enough DNA in order to achieve the cloning step. These difficulties could be explained by different raisons; RNA was insufficient in several samples because the initial amount was not enormous or RNA was degraded through time of conservation. For the sample Ange, an amplicon of 3043 bp was amplified with primer pair 1531-Linkerbis AUAP and clusters after sequencing with NV GIV.1. Few sequences are available in Genbank for this genotype and no complete genome is available. We will attempt to obtain the whole genome sequence. For one GII.4 2006b sequence, a newly designed primer pair (GII.4 NS1/NS3) was able to amplify segment of about 1500 bp

at the 5' end of the genome. Further work will be done to amplify various regions of the norovirus genome and might lead to the discovery of other putative recombination breakpoints.

3. Sequencing of NV strains detected in shellfish

Neither of the different sequencing attempts has given results because of the insufficient quantity of DNA or lack of specificity of the primer pairs. More attention will be given to the extraction procedure of the molluscan matrices. In the second part of the project, the extraction method using Nuclisens® minimag (Biomerieux) and its extraction reagents.

Work package 4: Development of a Network (P3)

In 2007, in total, about 100 food-borne outbreaks were reported to the NRL–VTI. In 11 outbreaks NV could be detected. Table 23 presents an overview of the different NV cases of 2007.

Table 23: An overview of the NV cases reported to the NRL-VTI in 2007.

Province	Persons ill	Matrix	Place	Genotype
ANT	74/135	chicken with rice –soup faeces	Recreation place	GGII GGII
HAI	35/325	mashed potatoes faeces	Elderly home	GGII Bacteria -
VBR	4/132	meat stew faeces	At work	GGII no samples
LIE	70	Mixed Faeces	Care center	GGII no samples
HAI	40/400	Sandwiches Faeces	Camp	No rest GGII
ANT	32/36	Sandwiches Faeces	Restaurant	GGII no samples
ANT	3	Chinese meal faeces	Restaurant	No leftovers GGII
LIE	40/105 (36hos)	water? Faeces	Camp	not detected GGI
WVL	49/200	Sandwiches Faeces	At work	GGII Bacteria -
ANT	16/72	Sandwiches Faeces	At work	GGI Bacteria – Personnel -
LIM	14	food? Faeces	Hospital	No rest GGII

In total 377 persons became ill after a NV infection. The symptoms of illness started in most of the cases between 12 and 24 hours after food consumption and in general vomiting, diarrhea and slight fever were reported. In almost all the cases hospitalization was not necessary. In two outbreaks it was clear that an infected person handling the food was at the origin of the further spread of the infection. Only in one case NV was detected both in the food and in the fecal samples taken. In the other outbreaks NV was not detected in the food or no leftovers were present to analyze. In other outbreaks no faecal samples were taken or those not send to the laboratory for NV detection. The most frequently implicated food item in these outbreaks was sandwiches. These were most likely contaminated by infected food handlers during preparation. Health care centers, at work, camp and restaurant visit were the most common settings.

In 2008, food-borne outbreaks were further followed up. In total 224 outbreaks were reported to the national reference laboratory and 884 people became ill. In 11 outbreaks Norovirus was detected as causative agent for the outbreak. In 5 of those 11 norovirus outbreaks food was not the source for the outbreak. Those outbreaks occur in elderly homes and for the transmission of the virus person to person contact is the most important we do not have the correct figures how many persons became ill. In the other 6 outbreaks food was suspected as possible source of the outbreak and samples were analyzed. In the first outbreak in the beginning of the year, 200 of 3000 exposed people became ill (diarrhea, vomiting and slight fever) after eating sandwiches at a new year's event. The outbreak took place at a recreation place where the families of a factory were invited to celebrate the beginning of the year. Faecal samples of different patients were taken but were bacteriological negative. The human samples analyzed in the routine clinical laboratory were send to the NRL-FBO and tested positive for Norovirus GII. Also leftovers of the sandwiches fillings were analyzed bacteriologically and for Norovirus but all were negative. The source of this Norovirus outbreak could be the sandwiches contaminated by a food-handler but not enough epidemiological information was available for making this conclusion. Otherwise person

to person spread could have been also an important factor in this outbreak. A second outbreak happens in July at a working place where 15 of the 70 exposed persons became ill after eating sandwiches. Different fillings were tested and three samples were tested positive (meatball, cheese and chicken curry). A copro culture of one persons helping in the kitchen to prepare the sandwiches was tested positive for Norovirus genotype GII. The food handler was forbidden to enter the kitchen area. After a month a new sample was taken and then he tested negative. In this case the food handler was proven to be the source of infection. A third outbreak happened in September where 80 persons became ill. Different groups became ill after having a barbecue which was prepared at the same butcher. Different leftovers of the food were tested but were negative. Different copro cultures were tested from infected persons and tested positive for Norovirus Genotype GII. The copro culture of the women of the butcher was tested positive. In the butcher's family there was a history of gastroenteritis the week before the outbreak. There were no remarks on the hygiene practices in the butcher's shop. In this case also the food handler was a very important in the transmission of the virus. Another outbreak occurred at a school trip were 47 children became ill. First we thought that the food was the source of the outbreak. After receiving more epidemiological information it was clear that one child has vomiting in the toilet and it was only cleaned with water without using a detergent. In Limburg a Norovirus outbreak was reported in a children's garden where 15 young children's became ill. They received the dinner prepared by an institutional catering who served also the dinner to different elderly homes in the same village. In those placed no infections occurred. So no further food analysis took place. But in this outbreak person to person transmission was the most important route. In the last outbreak 38 persons became ill after having a buffet dinner in a restaurant. Food samples and leftovers of the dinner were analyzed but are negative for norovirus. One of the clients also present in the restaurant suffered already from a gastroenteritis and maybe she was the source of the transfer to other people present in the restaurant. But for this we do not have any evidence.

A Norovirus outbreak on a cruise ship in Antwerp was reported but no faecal or food samples were taken. Only recommendations for cleaning and disinfection were asked.
For a better follow up of foodborne outbreaks two training days on Norovirus and foodborne outbreaks in general were organized for the officers of the Belgian food in Belgium. Together with the health inspectors of the communities a protocol was worked out for the follow up of the faecal samples in the frame of outbreak investigations.

V. PRELIMINARY CONCLUSIONS

Work package 1: Methods of analysis

The animal species to focus on for the development of a real time RT-PCR method to detect animal NV in stool samples were defined. Various animal species stools were screened with broadly reactive primers pairs. NV has only been detected in bovine and porcine faecal samples. Unfortunately, distinct primer pairs were necessary for the detection of bovine and porcine noroviruses. Other caliciviruses (sapovirus and feline calicivirus) were detected in porcine, canine and feline samples and these species need additional attention in the continuation of the project. All equine stool samples were negative with the primer pairs used. Other primers need to be investigated in order to completely exclude the existence of caliciviruses in this species and more particularly noroviruses. Calicivirus-like polymerase sequences were amplified from canine and poultry stool samples indicating the potential of a yet unknown calicivirus infecting these species.

A. Real time RT-PCR protocols have been evaluated for detection of GGI and GGII NVs. The use of the Taqman Universal Mastermix has been privileged in combination with the CEN/TC/WG6/TAG4 primers and probes. The methods were optimized using pGI and pGII plasmids as standard instead of single stranded DNA fragments to prevent contamination. Optical adhesive films were preferred to seal the 96-well plates to limit contaminations. The real-time PCR protocol for the detection of MNV-1 designed by Baert et al., 2008 (2) was shown to be appropriate for the detection of MNV-1. All singleplex assays were successfully tested on two different thermocyclers (ABI Prism® SDS 7000 and Roche Lightcycler

LC480). Analysis of the detection limit of the 3 individual assays showed that a minimum of 10 copies of the pGI/pGII/p20.3 plasmids – containing primers-probe binding sites of respectively GGI and GGII NVs and MNV-1 – were consistently detected at mean Ct values of respectively 37.38/38.02/35.11.

B. All optimized singleplex real-time PCR assays were combined into one multiplex assay and, when equally mixed amounts of the pGI, pGII and p20.3 plasmids were detected with the multiplex assay, only a negligible loss in sensitivity was noticed in comparison to the singleplex reactions.
When pGI, pGII and p20.3 plasmids were mixed in different concentrations, a mutual competitive effect was noticeable between the individual GGI and GGII reactions within the multiplex assay. This competitive effect became clear when a 2 log excess (10^5 / 10^3 copies and 10^3 / 10 copies) was present between the 2 targets (pGI/pGII), resulting in Ct-shifts between 1.8 and 5.6 Cts. Moreover, when a 4 log excess (10^5 and 10 copies) was present between the 2 targets (pGI/pGII), the target with the lowest concentration could not be detected (Ct>50).
The effect of the MNV-1 reaction on the GGI and GGII reactions within the multiplex assay was limited when pGI or pGII were solitarily present. However, the presence of 10^3 and 10^5 copies of p20.3 did cause Ct-shifts when a 2 log concentration difference between GI and GII was present.
This observation showed the limits of the multiplex assay for the detection of low amounts of one NV genotype (GGI/GGII) in the presence of high amounts of another NV genotype (GGII/GGI) in the same sample.
These results also indicated that the use of the MNV-1 reaction as PCR internal amplification control (IAC) is achievable. To avoid any competitive effects and to avoid the loss of the quantitative properties of the multiplex assay (especially when detecting low virus concentrations), no more than 10^2 to 10^3 copies of plasmid p20.3 should be added to the real-time PCR reaction as IAC when detecting GI/GII NoVs.

C. Specificity and sensitivity of the multiplex assay was analyzed by testing 16 clinical samples, a Norovirus RNA reference panel and 7 alternative viruses. All samples previously found positive for GGI or GGII NVs were also detected in the respective GI or GII PCR assays within the multiplex PCR. All tested genotypes present in the Norovirus reference panel were specifically detected. No cross amplification between the GI and GII genogroups was noticed. Negative samples and all alternative virus types tested negative.

The developed multiplex real-time RT-PCR assay is a specific and sensitive method for quantification of GGI and GGII NVs. Partner 3 will use and evaluate this assay for detection of NVs in clinical/food samples in case of suspected foodborne NV outbreaks.
Further development of a method for the detection of NVs in food matrices will include the development/optimization of the sample preparation: different protocols for the virus/RNA extraction on different food matrices (fresh produce/ready-to-eat foods) will be compared and evaluated. In this study, products will be spiked with MNV-1 and the extraction efficiency will be analysed by the developed real-time RT-PCR method.

The CEN WG6 TAG4 protocol has been tested on different shellfish matrices. A novel internal control was developed to avoid contamination. GGII was found predominant in the analyzed samples; this finding is concordant with data from other European countries. Detection limits of 35 particles per reaction and 25-250 particles per reaction for GGI and GII respectively were determined based on synthetic RNA. Ring-tests for NV detection in shellfish have been organized between the CEN members and situated the P5 laboratory within the best scoring.

Work package 2: Virus evolution

Genotyping of the detected NV strains in the different matrices is important to understand transmission routes of NV. Sequences available from the polymerase and capsid regions were obtained by direct sequencing of the RT-PCR products amplified in stools. All positive stools from outbreaks in 2007 provided by the IPH were GGII.4 variants 2006a and 2006b. These results would confirm other reports that, since their emergence in 2006, describe these variants as the most circulating strains worldwide (only the denomination changes over the continents; they are known as Laurens and Minerva in the USA, v4 and v6 in the UK). The two variants are thought to co-circulate in the same proportions but GII.4 2006b seems more prevalent in most European countries for outbreaks in season 2007-2008. Unfortunately the lack of positive samples in this study did not allow us to confirm this on a Belgian level. For 2008 this trend was not observed, GII.4 2006 variants were still implicated in outbreaks but other norovirus genotypes and genogroups were detected in stools from outbreaks and isolated cases of gastro-enteritis. One sample seemed to be co-infected by two different genogroups, namely GII and GIV. As co-infection could enhance recombination events, the sample will be further investigated to find out if there is evidence of recombination. One isolated gastro-enteritis case showed the presence of a sapovirus GI.2, this result could not be included in the risk analysis study for Belgium because the sample originated from a bordering region in France. No other sample was positive for the genus sapovirus.

Most of bovine NVs detected correspond to the GGIII.2 Newbury strain. The identification of several natural recombinant strains GIII.1/GIII.2 stipulates that not only human noroviruses are capable of recombination. Even if up to date there is no clear evidence that cattle can be infected by human noroviruses, this finding could maintain the question of the zoonotic potential of animal NV. Recombination events could engender the barrier crossing of these viruses especially in countries were cattle and human beings are in close contact. The detection of NV and SV closely related to human NV and SV strains in pigs fears for this potential zoonotic risk. Pigs are known to be experimentally capable of being infected by human NV but this statement could not be confirmed in the field. Recombination between human and porcine NV has not yet been described but should not be excluded.

Genotyping and the study of recombinant NVs require sequences that cover the ORF1/ORF2 junction and the whole capsid gene sequence. Thanks to the six fragments we were able to amplify, we can be sure that the sequences of the polymerase and the capsid region both issued from the same genome and that the samples did not seem to be co-infected by different strains. Unfortunately we did not succeed yet in amplifying a large fragment of the UCL5 and 6 samples. The sequences from the polymerase and the capsid regions of these samples did not cluster in the same genotype. A fragment of approximately 1000 bp covering the ORF1-ORF2 junction was amplified. The Simplot analysis indicated the breakpoint to be at the junction like it was expected confirming what was observed before. For most reported recombinant NV strains this breakpoint was also located in this junction.

Positive shellfish and food samples provided by P5 and P3 respectively were amplified for sequence analysis. A lack of material made it impossible to amplify enough exploitable DNA. The following phase will be fully consecrated to this purpose. Food samples were linked with outbreaks and even that there was not enough RNA in the samples. Inhibition in this kind of matrices is very important and the extraction method is a crucial step for the detection of viruses.

Work package 4: Development of a Network

Since the start of the reporting, the causative agent remains unknown in 20 to 50% of the reported food-borne outbreaks in Belgium. NV is suspected to be an important cause of food-borne outbreaks and could be responsible for a large part of these unknown cases. However up to now no robust extraction and detection system for the detection of this virus is available for routine analyses of different kinds of foodstuffs, neither there is an international approved isolation and detection method for NV in different kind of foods. Furthermore the procedures described in literature are not suitable for a routine analysis.

Moreover, in most of the cases no fecal samples of the patient are taken and in some cases there remain no leftovers of the food. So it is difficult to find the epidemiological link to trace back the contaminated food which has been the source of the infection. Also NV infections are underestimated because the infection is normally self-limiting in 24 h and complications are not known. Because of these shortcomings a better protocol was worked out with the doctors of the health inspections to send us the faecal material. Faecal material is much easier to analyze than food, because of the higher concentration of virus particles and already al lot of standard RNA extraction protocols available. However, it was also possible to detect Norovirus in different kind of foods by the procedure described by Baert et al. 2006. Now we are waiting for the optimized protocol of partner 4 to change our food extraction protocol to have an overall better sensitivity of the procedure.

VI. GENERAL CONCLUSIONS

Most of the objectives planned for the first 2 years of the project have been achieved as presented above. There are indeed several points that need to be improved in the second part of the project.

In WP 1 further studies should be realized on the extraction methods depending on the different matrices.

In WP 2 further progress should be made on the sequencing of viral genomes detected in shellfish.

The WP 3 was started during the first part and results are included in the WP 1 and WP 2. Epidemiological data was collected by P3 and P5. To evaluate the zoonotic potential of NVs, the identification of NV in animals was essential. Results from strain typing of these circulating NV will be used in the risk profiling.

For WP 4 the network has been established and needs to be implemented in the second part of the project.

VII. RECOMMENDATIONS

The Norisk network was already able to setup and apply a diagnostic procedure of NV detection of food matrices and human samples. The diagnostic procedures allowed the identification of several outbreaks of gastroenteritis. The application of this procedure allowed the identification of NV as the first cause of food-borne gastroenteritis in Belgium in 2007.

Therefore public health should be concerned by this diagnostic figure in Belgium and instructions should be given to professionals in order to reduce the risk of food contaminations and inter-human dissemination of the infection.

The recommendations arisen from Norisk scientific work are being distributed to the scientific and medical communities through the participation of the Norisk partners to several committees and working parties. All partners are members of the working group of the Belgian *Conseil Supérieur de la Santé (CSS) – Hogegezondheidsraad (HGR)* to study virus transmission by food. Partners 1 and 2 both participate in the European Network for Environmental and Food Virology (COST Action 929). Partners 3 and 5 are National Reference Laboratories of foodborne outbreaks and food virology respectively.

PUBLICATIONS

Baert, L., Uyttendaele, M., Stals, A., Van Coillie, E., Dierick, K., Debevere, J., Botteldoorn, N. **Reported foodborne outbreaks due to noroviruses in Belgium: the link between food and patient investigations in an international context.** *Epidemiol Infect., 2009, 9, pages 1-10.*

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Baert, L., Uyttendaele, M., Van Coillie, E., Debevere, J. (2008). **The reduction of murine norovirus 1, B. fragilis HSP40 infecting phage B40-8 and E. coli after a mild thermal pasteurization process of raspberry puree**. *Food Microbiol., 2008, 25 (7), pages 871-4.*

Baert, L., Uyttendaele, M., Vermeersch, M., Van Coillie, E., Debevere, J. (2008). **Survival and transfer of murine norovirus 1, a surrogate for human noroviruses, during the production process of deep-frozen onions and spinach**. *J. Food Prot., 2008, 71(8), pages 1590-7.*

Mauroy A., Scipioni A., Mathijs E., Thiry E., **Les norovirus bovins, virus entériques méconnus dans l'espèce bovine.** *Ann. Med. Vet., 2007, 151 (4), pages 257-268.*

Mauroy A., Scipioni A., Mathijs E., Miry C., Ziant D., Thys C., Thiry E. **Norovirus and sapovirus in pigs in Belgium.** *Arch. Virol., 2008, 153, pages 1927-1931.*

Mauroy A., Scipioni A., Mathijs E., Saegerman C., Mast J., Bridger J. C., Ziant D., Thys C., Thiry E. **Epidemiological study of bovine norovirus infection by RT-PCR and A VLP-based Antibody ELISA.** *Vet. Microbiol. Accepted for publication.*

Scipioni A., Bourgot I., Mauroy A., Ziant D., Saegerman C., Daube G., Thiry E. **A novel TaqMan RT-PCR assay able to detect and quantify human and bovine Noroviruses**. *Mol. Cell. Probe, 2008, 22, pages 215-222.*

Scipioni A., Mauroy A., Vinjé J., Thiry E. **Animal Noroviruses**. *Vet J., 2008, 178(1), pages 32-45.*

Scipioni A., Mauroy A., Ziant D, Saegerman C., Thiry E. **A SYBR Green RT-PCR assay in single tube to detect human and bovine noroviruses and control for inhibition.** *Virol. J., 2008, 5:94.*

Scipioni A., Mauroy A., Mathijs E., , Ziant D., Daube G., Thiry E. **Molecular analysis of contemporaneous human and bovine noroviruses in Belgium**. *Submitted for publication.*

Stals, A., Werbrouck, H., Baert, L., Botteldoorn, N., Herman, L., Uyttendaele, M. en Van Coillie, E. **Laboratory efforts to eliminate contamination problems in the real-time RT-PCR detection of Noroviruses.** *Accepted for publication.*

Stals, A., Werbrouck, H., Baert, L., Botteldoorn, N., Wollants, E., Herman, L., Uyttendaele, M., Van Coillie, E. **Multiplex real-time RT-PCR for simultaneous detection of GI/GII Noroviruses and Murine Norovirus 1.** *Submitted.*