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TRANSMISSION ROUTES OF NOROVIRUSES, EMERGING HUMAN PATHOGENS IN FOOD "NORISK"

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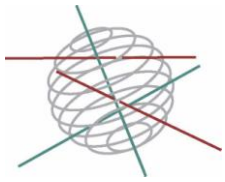

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FINAL REPORT

TRANSMISSION ROUTES OF NOROVIRUSES, EMERGING HUMAN PATHOGENS IN FOOD "NORISK"

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SUMMARY

A. Context

Noroviruses are pathogens causing gastroenteritis and infections result in typical symptoms such as abdominal cramps, fever, watery diarrhea and other symptoms such as headaches, chills and general myalgias, which usually last for 2 to 3 days. The illness is self-limiting in most cases. The NV genus contains 5 genogroups whereby genogroup I and II (GI and GII) comprise most of the human infective NV genotypes. Bovine and murine NV are classified respectively in genogroup III (GIII) and V (GV), while porcine NV are also classified in GII. Human infective (mainly GI and GII) noroviruses (NV) have increasingly been recognized as a global major cause of acute non-bacterial gastroenteritis, but sensitive detection is only possible by molecular methods, due to the unavailability of a cultivation system. Development of these molecular methods showed that NV could be responsible for 60 % and 77 % of all gastroenteritis cases with known etiology in the USA and in Europe, respectively. The fraction of NV outbreaks caused by consumption of contaminated foods is estimated to be 10 to 20 %. Food products can be contaminated through 2 main transmission routes: either pre-harvest contamination, whereby mostly fresh produce and bivalve shellfish are involved. Shellfish are contaminated by cultivation in contaminated water, while fresh produce can by contaminated by use of contaminated irrigation water or (post-) harvest contamination often involving an infected food handler or food picker. A broad range of food products are related to the latter transmission route. Detection of NV in foods is more difficult because detection of NV present at very low levels on the foods should be possible due to the low infectious dose. Therefore, (genomic material of) NV has to be extracted from the foods and has to be detected subsequently by a molecular detection method. Furthermore, NVs are present in several animal species, raising important questions about zoonotic transmission and potential animal reservoir.

B. Objectives

1. The NV 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 RT-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 seafoods and with emphasis on elaboration of an appropriate extraction producedure in fresh produce/ready-to-eat foods.
3. The routine detection of NVs in food stuffs (seafoods 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

C. Conclusions

Objective 1:

A multiplex real-time RT-PCR assay for simultaneous detection of human GI and GII NV in clinical samples was designed, with the successful inclusion of MNV-1 as real-time PCR IAC. Evaluation of this multiplex assay showed a high concordance between the multiplex assay and the corresponding singleplex PCR assays. Specificity analysis of the multiplex assay by testing a NV RNA reference panel and clinical GI and GII NV samples showed that specific amplification of NV GI and GII was possible. In addition, no cross-amplification was observed when subjecting a collection of bovine NV and other (non-NV) enteric viruses to the multiplex assay. Finally, MNV-1 was successfully integrated as IAC, although a sufficiently low concentration was needed to avoid interference with the possibility of the developed multiplex assay to quantitatively and simultaneously detect the presence of GI and GII NV within one sample.

Persistent contamination problems leading to false-positive results were encountered, but an investigation was performed towards the source of the contamination. The problem could be controlled and only occasional contamination has been observed.

Objective 2:

Two protocols for extraction of NV from soft red fruits (selected as fresh produce product) and ready-to-eat (RTE) foods were evaluated towards robustness and sensitivity.

For the RTE foods, the protocol for RTE foods made use of a guanidine isothiocyanate containing reagent to extract viral RNA from the food sample (basic protocol called TriShort) with an eventual concentration/purification step (extended protocol called TriConc). The protocol for extraction of NV from soft red fruits consisted of alkaline elution of NV particles from the food, followed by polyethylene glycol precipitation and organic solvent purification. After purification, the RNA was detected by the multiplex real-time RT-PCR assay optimized in objective 1. The influence of (1) the NV inoculum level and (2) different food types on the recovery of NV from these foods was investigated for both protocols.

Overall, the elution –precipitation protocol was able to recover NV from soft red fruits with efficiencies of 10 % to 20 % in most cases while the protocol for RTE foods yielded recovery efficiencies of >1% (TriShort protocol) and 0.1 to 10 % (TriConc protocol). For both NV extraction methods, taking into account all dilution factors resulted in a detection limit of approximately 10^4 genomic copies/10g. Simultaneous recovery of GI and GII NV in similar or 100-fold different concentrations was possible in both food categories.

A significant influence of the NV inoculum level on its recovery was noticeable in both protocols as high inoculum levels were recovered more successfully and with a higher efficiency compared to low level inocula in both protocols. This phenomenon, together with the influence of the food type on the recovery was more explicit on the protocol for RTE foods compared to the protocol for soft red fruits.

Objective 3:

The multiplex real-time RT-PCR assay described in objective 1 and the virus extraction protocols described in objective 2 were combined to two NV detection methods. The murine norovirus 1 (MNV-1), a cultivable genogroups V NV, was in these detection methods used and evaluated as control reagent. MNV-1 was used to control the entire virus detection protocol (process control; PC), the reverse transcription reaction (reverse transcription control; RTC) and the real-time PCR reaction (internal amplification control; IAC) when detecting NV in foods. Evaluation showed that MNV-1 PC and RTC could be used for detection of inefficient extraction and inhibition of the RT-PCR, respectively. On the other hand, the MNV-1 IAC provided only little added value and it was suggested to leave this control out.

Objective 4:

Screening of 75 fruit samples for NV presence was performed using the protocol for soft red fruits (objective 2) and the multiplex real-time RT-PCR assay (objective 1). MNV-1 was used as PC, RTC and IAC. A total of 18 samples tested positive for GI and/or GII NV despite good bacteriological quality. Results obtained showed the difficulty of expressing positive (real-time) PCR results towards terms of public health threat if no associated diseases or outbreaks are reported. Although these low NV levels might

indicate virus contamination at some point during the fresh produce chain, care should be taken to translate these results as a significant risk to the public health. Nevertheless, a possible risk for food borne transmission of NV from these food products cannot be excluded either.

Genotyping results from 115 clinical samples originating from gastro-enteritis epidemics reported to the Scientific Institute of Public Health allowed us to characterise the NV strains implicated in these outbreaks between 2007 and 2010. Similarly, the creation of a stool bank with domestic animal clinical samples and NV screening in these samples in the first part of the NORISK project have allowed the characterisation of animal NVs especially in the bovine and porcine species. These results confirm that bovine and porcine NVs may be endemic in our counties but besides these animal NVs, no other animal NV was detected in the other animal species selected for the stool bank.

Objective 5:

After the introduction of Norovirus specific analysis method in the surveillance of foodborne outbreaks, it became clear, that Norovirus is an important agent causing foodborne outbreaks in Belgium. During the last three years it was even the leading reported agent. It became also clear that it is not so easy to define the transmission routes of Norovirus. By the introduction of a scenario for gastro-enteritis a classification based on the possible transmission route was possible. In all the reported outbreaks no primary contaminated food like bivalve shellfish or red fruits was involved. Secondary contaminated food plays an imported role in the transmission of Norovirus with an infected food handler as a crucial vector. Besides the food related outbreaks it became clear that person-to person transmission and a high environmental contamination are risk factors in the further transmission of Norovirus in the population The fact that many people are living close together in for example youth camps or elderly homes, the common use of sanitary facilities and the common preparation of meals, combined with the high infectivity of Norovirus and the existence of asymptomatic carriers, results in highly vulnerable populations in these conditions. Although Norovirus infections often end up in a positive way, they may have a high impact on the health (eg elderly homes) and may cause a lot of costs (less personnel at work) and sorrow (eg closure of a youth camp). Although both the prevention and decrease of the risk of a Norovirus infection are not evident, some measures have to be taken. A good hand-, toilet- and kitchen hygiene, a good infrastructure and the rapid signaling of gastro-enteritis outbreaks can decrease the risk of Norovirus infection and might restrict further spread of the virus. The knowledge rising from the Norovirus outbreaks reported at the NRL FBO allowed use to formulate and publish specific measures and recommendations for Norovirus outbreaks, which help the inspectors and physicians in the rapid diagnosis and prevention of the further transmission of Norovirus outbreaks.

Objective 6:

Throughout the NORISK project, NVs were detected in different food matrices available for human consumption, in humans and in animals like cattle and pigs. For a better comprehension of NV transmission routes, sequences of the detected NVs were determined and submitted for further analysis. Genotyping of NVs in food matrices came out to be a real challenge and consisted into a bottleneck as the amount of genetic material on food was insufficient for PCR amplification and sequencing. This obstacle was not overcome during our project and NV sequences were only obtained from clinical samples in humans and animals. Interesting was that no animal NVs were detected in samples originating from humans and no human sequences were amplified from animal clinical samples. Thus, there is no evidence of a potential interspecies NV transmission and zoonotic transmissions seem unlikely to occur. However NV, being an RNA virus, exhibit great genomic plasticity and changes in its genome could lead to the emergence of new NV variant with different biological proprieties that should not be left out (objective 7).

Objective 7:

Sequences obtained in the human and bovine clinical samples show different NV strains that exhibit incoherent clustering for the partial sequences of the polymerase and the capsid region indicating that they might be recombinant. For the human NV strains, although the majority of the gastroenteritis outbreaks were involved with GII.4 NVs in 2007 and 2008, other GII NVs were detected from the end of 2008 to 2010 along GII.4 NVs. Among these NVs, a variety of new recombinants were detected in different samples from different outbreaks between 2008 and 2010. New « super » polymerase sequences (GII.e and GII.g) related to the previously described GII.b polymerase were detected in the same period. The exact significance of the emergence of these polymerases or their origin has yet to be elucidated but their involvement with different outbreaks might indicate that they have a selective advantage upon the capsid parental strains.

Based on sequencing data, norovirus (NV) recombinants have been described, but no experimental evidence of recombination in NVs has been documented. Using the murine norovirus (MNV) model, we investigated the occurrence of genetic recombination between two co-infecting wild-type MNV isolates in RAW cells. The design of a PCR-based genotyping tool allowed accurate discrimination between the parental genomes and the detection of a viable recombinant MNV (Rec MNV) in the progeny viruses. Genetic analysis of Rec MNV identified a homologous-recombination event located at the ORF1–ORF2 overlap. Rec MNV exhibited distinct growth curves and produced smaller plaques than the wild-type MNV in RAW cells. Here, we demonstrate experimentally that MNV undergoes homologous recombination at the previously described recombination hot spot for NVs, suggesting that the MNV model

might be suitable for in vitro studies of NV recombination. Moreover, the results show that exchange of genetic material between NVs can generate viruses with distinct biological properties from the parental viruses.

D. Keywords

Human norovirus, animal norovirus, molecular detection, multiplex real-time RT-PCR, murine norovirus 1, in vitro recombination, virus extraction, soft red fruits, ready-to-eat foods.

1. INTRODUCTION

The viral family *Caliciviridae* is divided into four genera: Vesivirus, Lagovirus, Norovirus and Sapovirus. Norwalk virus is the prototype strain of genetically and antigenically diverse single-stranded RNA viruses belonging to the Norovirus genus which was first described in 1972 in association with an outbreak of gastroenteritis and vomiting involving children and staff at an elementary school in Norwalk, Ohio (49, 50). Noroviruses (NVs) can be divided into at least five distinct genogroups: GGI and GGII contain most of human NVs, the porcine NVs fall within GGII; the bovine caliciviruses cluster into a proposed GGIII; two human NVs (strains Alphatron and Lauderdale) are proposed in GGIV; the recently described murine NV forms a proposed GGV (43).
These NVs are of great interest for Public Health and Food Safety. They are involved in many food borne outbreaks of different sources (shellfish, water, fruits for example). In addition, they are present in different animal species and animal strains are genetically so close to human NVs that a zoonotic hypothesis has raised but is still not elucidated. Another important phenomenon involved in NVs evolution is recombination. This mechanism can be studied on the basis of gene sequencing of isolated strains and comparison with published sequences from defined genotype/genogroup strains.

Human infective (mainly GI and GII) NV have increasingly been recognized as a global major cause of non-bacterial gastroenteritis (55). The spread of this pathogen is facilitated by a low infectious dose (10 to 100 infectious virus particles) combined with a high environmental stability (3, 121). The Norwalk viral agent (later renamed NV GI.I) was discovered in 1972 by Kapikian et al. in faecal specimens originating from a gastroenteritis outbreak in Norwalk, Ohio (50). Nevertheless, detection of NV has been hampered by lack of a cultivation method. Sensitive detection of NV has been possible since the 1990's, when the cloning and sequencing of the NV genome cleared the way for development of more sensitive molecular NV detection methods.
This development lead to the estimation that NV could be responsible for 60 % and 77 % of all gastroenteritis cases with known aetiology in the USA and in Europe, respectively (77, 127). The fraction of NV outbreaks caused by consumption of contaminated foods is estimated to be 10 to 20 % (57, 127, 133). In Belgium, NV has surpassed Salmonella as the most important food borne pathogen since 2009 (6)(Nadine Botteldoorn, personal communication). Food products can be contaminated through 2 main routes. Food products can be contaminated pre-harvest by contaminated irrigation or growth water whereby respectively fresh produce and bivalve shellfish are involved. A second main transmission route is through contact with an infected food handler or food picker and involves a broad range of food products.

A problem when investigating NV food borne outbreaks is that studies are mostly based on epidemiological and clinical data since the confirmation of food as source of a food borne outbreak is difficult to date. Detection of NV in foods is more difficult compared to most food borne bacterial pathogens, because cultivation is not possible thus far and because detection of NV present at very low levels on the foods should be able due to the low infectious dose. Therefore, (genomic material of) NV has to be extracted from the foods and has to be detected subsequently by a molecular detection method. Due to the complexity of these methods and the possibility of inhibition or reduced detection efficiency because of the food matrix, controls are required to assure the reliability of the obtained results.

Furthermore, several animal enteric calicivirus morphologically indistinguishable from the human caliciviruses and genetically closely related to NV have been reported (25, 38, 61, 118). Until that time, NVs were thought to be restricted to human. The first bovine NVs were described in England and are known as Newbury agent 1 and 2 (NA1 and NA2) (137). The bovine enteric CV (calicivirus) (Jena and Newbury viruses) represent two distinct clusters of GGIII NV (25, 61), while the swine enteric CV, that are closely related to human NV, represent a distinct cluster within GGII (118, 119). Like the human viruses, bovine NVs do not grow in cell culture. The discovery of these animal NVs raised important questions about zoonotic transmission and potential animal reservoir.
The epidemiology and prevalence of animal NV are not well understood neither. In the Netherlands, 31.6% of pooled stool specimens from veal calf farms and 4.2% of individual stool specimens from dairy cattle were positive for NVs related to Newbury virus (124). By using an ELISA specific for Jena virus, a study in Germany showed that 9% of diarrhea stool samples and 99% of the serum samples collected from dairy cows were positive for Jena virus antigens or antibodies, respectively (28). SW918, a prototype strain of swine NVs that shares 64 to 69% amino acid identity with other cluster representatives within GGII NVs, was first detected in the caecum contents of healthy pigs in Japan by RT-PCR in 1997 (118).The detection rate of swine NVs in Japan was low (0.35%), and a similar low detection rate (2%) was reported in The Netherlands (125). Recently the detection of swine NVs in 5 of 275 fecal samples collected from six pig farms in the United States was reported and by redesigning their original primers the detection rate improved to 23% (63/275) (131). In that study they identify a potential recombinant between pig NVs. At present NV recombinant have been detected exclusively between viruses in the same genogroup and within the same host species, but few animal NVs have been sequenced (RNA dependant-RNA polymerase (RdRp) and capsid), from comparative analysis, especially those from animals in developing countries, where humans and animals may be in close contact.

The classification of animal caliciviruses within the NV genus raises the question of whether zoonotic infections occur. Answering this question is important, because cross-species infections would affect the epidemiology and evolution of these viruses and complicate our ability to block transmission by vaccination or other therapy (95). In other animal species (horses or domestic carnivores for example), there was no NVs detected until now.
On another hand, recombination is an important mechanism in the evolution of RNA viruses since it can create changes in virus genomes by exchanging sequences, thus generating genetic variation and producing new viruses.
It is well documented that recombination occur quite often among NVs and thus contributes to the genetic diversity of these viruses (1, 12, 94, 131). Recombinant strains may be underestimated by the fact that NV characterization is usually based on the RdRp gene sequence only, whereas it is necessary to know both the RdRp and the capsid gene sequences to identify such viruses. Identification of recombination breakpoints in the region located at the junction of ORF1/ORF2 confirms the importance of this region in this phenomenon (12). It is important to work further on this topic because there is a need to determine more precisely the exact implication of recombinant NVs in gastroenteritis as well as basis for the selective advantage observed for some of these strains.

The aim of this project was 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 focused on the development of appropriate real-time RT-PCR for either detection or genotypic analysis of NVs. Subsequently the isolated strains were 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 were collected. The study of the various strains of NVs circulating in man and animals should make it possible to be proactive and to assess the risk of an emerging pathogen. A methodology, applicable in scientific laboratories of Belgian authorities and research institutes, was made available. The molecular epidemiology of detected viruses was of a major interest to trace the outbreaks from the source to sick people, geographically to know the circulation of the various strains of NV and within the population.

The objectives of this project were:

1) The norovirus RNA detection methodology: elaboration, optimization and evaluation of a real-time PCR format and determination of specificity, sensitivity and robustness. Two protocols were 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 seafoods and with emphasis on elaboration of an appropriate extraction procedure in fresh produce/ready-to-eat foods.
3) The routine detection of noroviruses in food stuffs (seafoods 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 noroviruses 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 noroviruses: genetic profiles and emerging of recombinants.

2. METHODOLOGY AND RESULTS

The work methodology was divided into different work packages as followed:

- Work package 1: Methods of analysis
    - Development and evaluation of molecular methods (real-time RT-PCR) for detection of human NV and animal NV. The specificity, sensitivity and robustness of the developed/optimized molecular methods would be analyzed. For this purpose, only NV clinical samples would be used as food samples need an additional virus extraction step.
    - Development of methods for extraction and concentration of NV (= sample preparation) from a broad range of food products. For the foods, focus was set to those foods that have been frequently involved in NV food borne outbreaks, such as fresh produce, ready-to-eat (RTE) foods and bivalve molluscan shellfish.
    - After evaluation and optimization of methods for extraction and molecular detection of NV in soft fruits, RTE foods and in shellfish, development and implementation of a standard protocol with establishment of appropriate controls for rapid screening of foods (fresh produce and bivalve molluscan shellfish) for the presence of NV. For both protocols, this objective included the evaluation of the murine norovirus 1 (MNV-1) as control reagent at different steps.
- Work package 2: Virus evolution
    - Genotyping and study of recombinant viruses in humans and animals
    - Recombination study of a cultivatable model virus for NV: MNV-1
- Work package 3: Risk profiling
    - The developed and evaluated NV detection methods (both for human and animal NV) and genotyping results were subsequently applied for the elucidation of transmission routes of NV through molecular tracing, with a global view on NV strains circulating among human, animal and also in food
- Work package 4: Development of a network

Work package 1: Methods of analysis

DEVELOPMENT AND EVALUATION OF MOLECULAR METHODS FOR DETECTION OF HUMAN NOROVIRUSES AND ANIMAL NOROVIRUSES

For the first objective of the NORISK project, real-time RT-PCR was selected. Most important reasons for this choice were i) the low detection limit in comparison to conventional RT-PCR and other molecular methods (10), ii) the absence of post-PCR processing and finally iii) the possibility of quantification (67, 88). The low detection limit (≤ 10 target copies) of this molecular detection method is necessary because of the (presumed) low viral concentration in environmental and food samples as well as because of the low infectious dose of NV. Therefore, real-time RT-PCR assays are considered to be the gold standard for detection of NV in clinical, food and environmental samples, (4, 46, 98, 136).

To control the reverse transcription and real-time PCR reaction, MNV-1 genomic ssRNA and a plasmid containing the full MNV-1 genome were used as reverse transcription control (MNV-1 RTC) and real-time PCR internal amplification control (MNV-1 IAC), respectively. To reduce the cost and hands-on time and in particular to take the option to include the mentioned MNV-1 RTC and MNV-1 IAC, a multiplex real-time RT-PCR assay was optimized.

For detection and quantification of GI and GII NV, primers and hydrolysis probes designed by the European Committee for Standardization/Technical Committee 275/Working Group 6/Task Group 4 on virus detection in foods (CEN/TC275/WG6/TAG4 working group) (60) were used. The primers and probe for detection and quantification of MNV-1 were designed by Baert et al. (7). An overview of the primers and probes sequences is shown in Table I. Primers and probes in all three singleplex assays targeted the ORF1-ORF2 junction regions (ORF: open reading frame), which are considered to be the most conserved region of the NV genome (47, 71, 89).

To achieve this first objective, the multiplex real-time RT-PCR assay for simultaneous detection of GI and GII NV and MNV-1 was optimized and thoroughly evaluated. The kinetics of the singleplex and multiplex assays were investigated, followed by a specificity analysis of the multiplex assay.

Table I. Overview of primers and hydrolysis probes used for real-time RT-PCR detection of GI and GII NV and MNV-1.

Primers/probes	Sequence (5' – 3')[a]	Polarity[b]	Position[c]	Final conc	Fluorophore[d] (5')/ Quencher (3')
NV GI					
QNIF4	CGCTGGATGCGNTTCCAT	+	5291-5308	500 nM	
NV1LCR	CCTTAGACGCCATCATCATTTAC	-	5354-5376	900 nM	
NVGG1p	TGGACAGGAGAYCGCRATCT	+	5321-5340	100 nM	6-FAM/BHQ-1
NV GII					
QNIF2	ATGTTCAGRTGGATGAGRTTCTCWGA	+	5012-5038	500 nM	
COG2R	TCGACGCCATCTTCATTCACA	-	5100-5080	900 nM	
QNIFS	AGCACGTGGGAGGGCGATCG	+	5042-5061	250 nM	Texas Red/BHQ-1
MNV-1					
FW-ORF1/ORF2	CACGCCACCGATCTGTTCTG	+	4972-4991	200 nM	
RV-ORF1/ORF2	GCGCTGCGCCATCACTC	-	5064-5080	200nM	
MGB-ORF1/ORF2	CGCTTTGGAACAATG	+	5001-5015	200nM	NED/MGBNFQ

[a] Mixed bases in degenerate primers and probes are as follows: Y, C or T; R, A or G; N, any;
[b] +, virus sense; -, anti-virus sense
[c] Corresponding nucleotide position of Norwalk/68 virus (accession nr. **M87661**) for NoV GI, Lorsdale virus (accession nr. **X86557**) for NoV GII or murine norovirus 1 clone CW1 (accession nr. **DQ285629**).
[d] BHQ-1: Black Hole Quencher – 1, MGBNFQ: Minor Groove Binding Non-Fluorescent Quencher

First of all, kinetics of the singleplex PCR reactions for detection of GI NV, GII NV and MNV-1 were investigated (Fig 1A). Analysis of the parameters of the standard curves of replicates of two independent runs showed that the three singleplex assays (GI NV, GII NV and MNV-1) were sensitive, as detection limits of 10 copies of the positive control plasmids containing primers-probe binding sites of GI NV (plasmid name: pGI), GII NV (plasmid name: pGII) and MNV-1 (plasmid name: p20.3) were observed. Moreover, high PCR efficiencies for the GI NV (91.6 %), GII NV (87.3 %) and MNV-1 (94.2 %) reactions were noticed and standard deviations were small (with a maximum of 0.92 Ct). Finally, the square regression coefficient (R2) value was $\geq$ 0.998 for all three singleplex assays in the concentration range tested and all intercepts were within a 2.9 Ct range. Other authors have reported similar detection limits ranging between 1 and 10 genomic copies. It is important to mention that the combination of these three singleplex reactions into a multiplex assay required similar PCR kinetics (81, 102). PCR-efficiencies of all singleplex assays were within a 9% range and intercepts differed less than 2.9 Cts.

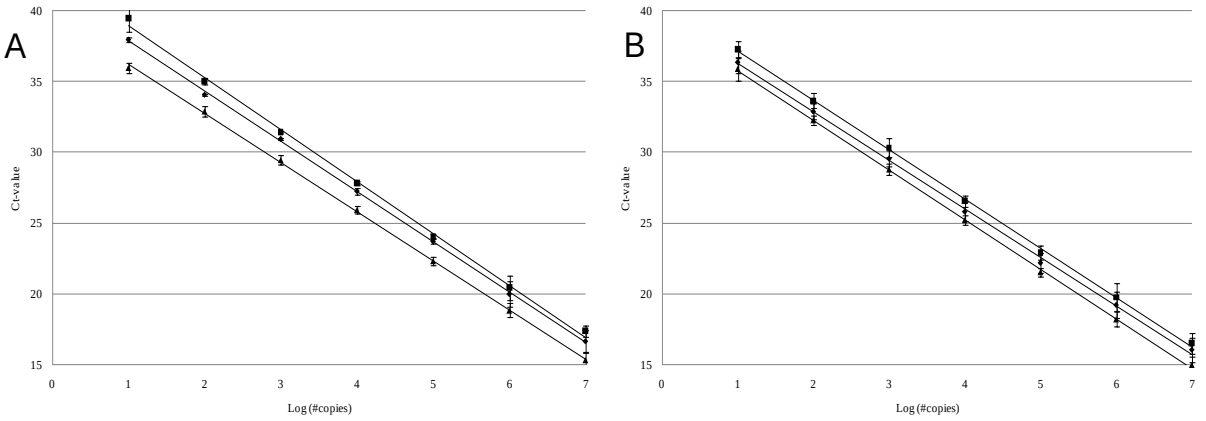


Fig. 1A: Standard curves for the three singleplex GI, GII and MNV-1 real-time PCR detection assays using 10-fold serially diluted plasmid standards of pGI (◆) , pGII (■) and p20.3 (▲), respectively, ranging from 10^7 to 10 copies.
Fig. 1B: Standard curves for the three individual GI, GII and MNV-1 real-time PCR detection reactions within the multiplex real-time RT-PCR detection assays using 10-fold serially diluted plasmid standards of pGI (◆), pGII (■) and p20.3 (▲), respectively, ranging from 10^7 to 10 copies.

Secondly, the effect of the multiplex setup on the individual PCR reactions for detection of GI NV, GII NV and MNV-1 was analyzed (Fig 1B). For this, plasmids pGI, pGII and p20.3 were each 10-fold serially diluted in water and subjected to the multiplex real-time PCR assay. Results showed that equally mixed amounts of the pGI, pGII and p20.3 plasmids could be detected with the multiplex assay, as only a negligible loss in sensitivity was observed in comparison to the singleplex reactions. In detail, all parameters of the standard curves of the individual GI, GII and MNV-1 reactions within the multiplex PCR indicated that these individual reactions were sensitive (detection limit of 10 copies) and efficient (PCR efficiencies of 96.1 %, 93.8 % and 93.1 %). Again, standard deviations were small (with a maximum of less than 1 Ct), and the R^2-value was $\geq$ 0.999 for all three individual PCRs within the multiplex assay in the concentration range tested. Lastly, all intercepts were within a 1.5 Ct range. This analysis showed that reliable detection of equimolar concentrations of all three target sequences was possible using the multiplex assay.

Thirdly, the competitive effect between the individual PCR reactions within the multiplex assay was tested by preparing all possible combinations of quantities of 0, 10, 10^3 and 10^5 copies of pGI, pGII and p20.3 and submitting these combinations to the multiplex assay. The resulting Ct values are shown in Fig. 2. A mutual competitive effect was noticeable between the individual GI and GII NV reactions within the multiplex assay. This competitive effect became clear when a 2 log concentration difference (10^5 /

10^3 copies and 10^3 / 10 copies) was present between the two targets (pGI and pGII), resulting in Ct-shifts between 1.8 and 2.9 Cts for the target present in the lowest concentration. Additionally, when a 4 log concentration difference (10^5 / 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 GI and GII 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 additional Ct-shifts when both pGI and pGII were present in one sample.

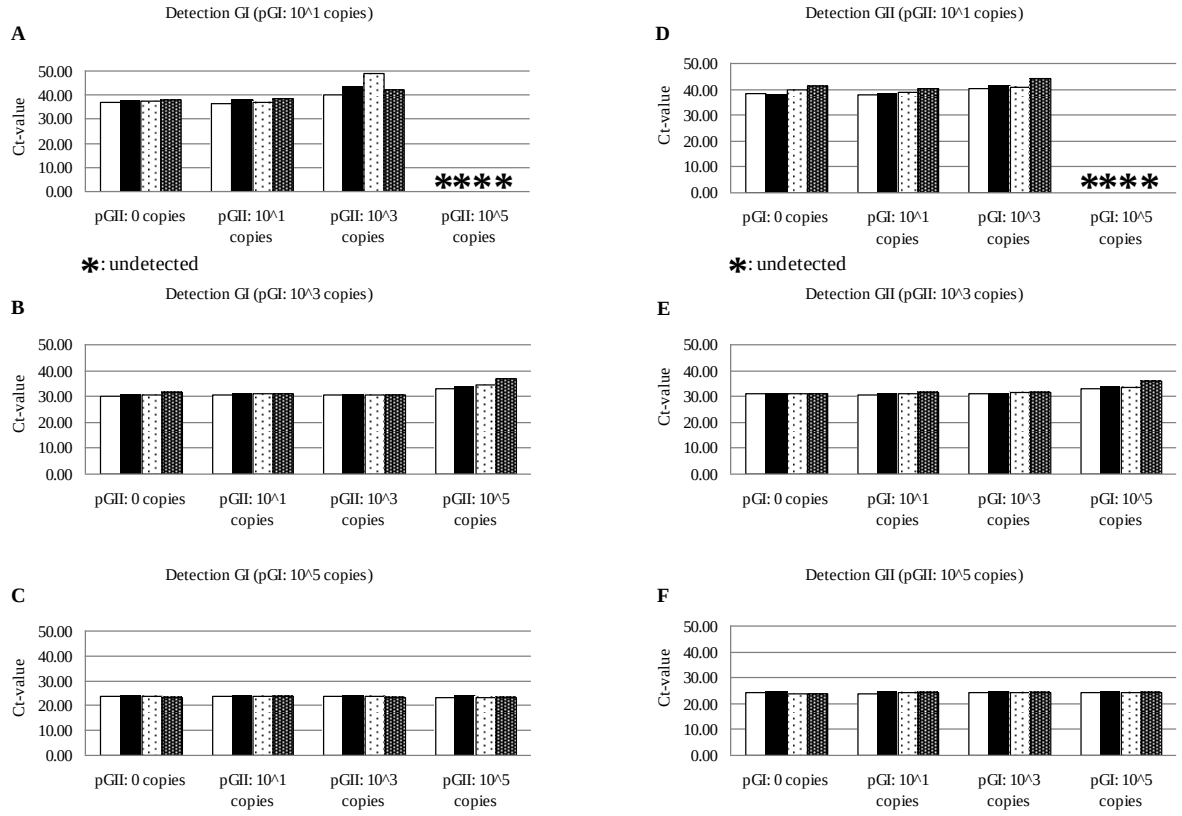


Fig. 2 A-B-C: The effect of the presence of GII on Ct values (vertical axis) of the GI reaction within the multiplex real-time PCR assay. Different copy numbers (0, 10, 10^3 and 10^5 copies) of pGII (horizontal axis) are combined with 10 (Fig 3A), 10^3 (Fig 3B) and 10^5 (Fig 3C) copies of pGI.

Fig. 2 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 3A), 10^3 (Fig 3B) and 10^5 (Fig 3C) 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 figure 3. Copy numbers of 0 (series □), 10 (series ■), 10^3 (series ⊡) and 10^5 (series ▩) of p20.3 were combined with any combination of copy numbers of pGI and pGII. All Ct values are means of duplicates.

This analysis of competition between the individual reactions within the multiplex assay showed the limits of the multiplex assay for the detection of low amounts of one NV genotype (GI/GII) in the presence of high amounts of another NV genotype (GII/GI) in the same sample. These results also indicated that the use of the MNV-1 reaction as real-time PCR internal amplification control (IAC) is achievable. To avoid (1) competitive effects and (2) 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 real-time PCR IAC when detecting GI/GII NV.

A previous study of competitive effects between individual reactions within a multiplex PCR assay designed to simultaneously detect 4 virus types did not report analogous Ct-shifts (81), but in this study only a 3-log difference between the target DNAs was investigated. However, the results of the current experiments support another multiplex real-time RT-PCR study (14), in which Ct-delays (2 to 3 Cts) were reported when low concentrations of viral genome (50 to 10^3 genomic RNA copies) were detected simultaneously with another abundant viral genome (10^4 to 10^6 genomic RNA copies). Competition of individual PCR reactions within a multiplex (real-time) PCR is a known problem (22) and the results of the current study show that this issue should not be neglected during the design and optimization of quantitative multiplex real-time PCR assays, especially when detecting low-concentration DNA targets. Nevertheless, a well-optimized multiplex (real-time) PCR assay has benefits, including reduced expense of reagents and preparation time, combined with the possibility to include a (real-time) PCR IAC (34).

After evaluation of the kinetics of the multiplex real-time PCR, the specificity of this assay was investigated by subjecting a Norovirus RNA Reference Panel containing in vitro synthesized ssRNA fragments covering genomic regions A, B and C (130) of nine GI, eight GII and one GIV NV (all human infective NV genotypes) and 16 clinical GI/GII NV samples to this assay (Table II). All tested genotypes in the Norovirus RNA reference panel were detected specifically, all clinical samples found positive for GI (5 samples) or GII (11 samples) NV previously were confirmed and no cross-amplification between the different GI, GII and GIV genotypes was observed. The seven alternative virus strains and the bovine GIII NV were not detected.

Specific detection of human NV genotypes by real-time RT-PCR has been demonstrated before in other studies (78, 136). However, the Alphatron (GIV) NV genotype was only included in a limited number of studies (46, 104, 123) and GIIc NV genotypes.

The use of MNV-1 as IAC was evaluated by adding 10^3 copies of the p20.3 plasmid to the real-time PCR reaction. This IAC was detected at the expected Ct value (~28), suggesting that no PCR inhibitory components were present in the cDNA preparations of the clinical samples.

Multiplex real-time RT-PCR assays for the simultaneous detection of GI and GII NV have been reported before (46, 47, 97) and many authors have suggested the use of a PCR IAC to detect possible false-negative results due to inhibition when detecting genomic material (42, 103, 112). This inhibition of (real-time) PCR assays is a known problem when detecting pathogens in faecal samples (58, 82, 92), sewage samples (39) and food matrices (105). Therefore, a cultivable MS2 bacteriophage (31, 107), a genetically modified cultivable mengovirus (21) and a cDNA fragment (whether or not NV-related) flanked by primer binding sites (35, 112) have recently been used as (multiplexed) PCR IAC. The use of DNA originating from a cultivable surrogate as (multiplexed real-time) PCR IAC is favored above the use of a cDNA fragment flanked by primer binding sites, as these cultivable surrogate organisms can also be utilized as (quantifiable) process control for the full extraction procedure when detecting GI and GII NV in clinical, environmental and food samples. A comparison between several cultivable NV surrogates for the detection of human NV in water favored the use of MNV-1 compared to other candidates such as MS2 bacteriophage, feline calicivirus (11) and poliovirus (2). In addition, the similar biological properties of MNV-1 and human GI and GII NV (15, 134) make it a preferred process control in human NV detection assays.

Table II. Overview of all tested samples/RNA fragments.

Virus type / NV genotype	Sample type	Source	Ct GI	Ct GII	Ct MNV-1
GI.?	Faeces	IPH[a]	38.46	Undet	27.89
GI.1 (Norwalk)	RNA fragment	RIVM[b]	29.01	Undet	Undet
GI.2 (Whiterose)	RNA fragment	RIVM	20.52	Undet	Undet
GI.2	Faeces	REGA[c]	29.79	Undet	27.70
GI.2	Faeces	REGA	28.07	Undet	27.69
GI.2 (Southhampton)	RNA fragment	RIVM	20.83	Undet	Undet
GI.3 (Birmingham)	RNA fragment	RIVM	19.09	Undet	Undet
GI.4 (Malta)	RNA fragment	RIVM	19.33	Undet	Undet
GI.4	VTM[d]	REGA	26.04	Undet	27.56
GI.5 (Musgrove)	RNA fragment	RIVM	39.12	Undet	Undet
GI.6 (Mikkeli)	RNA fragment	RIVM	19.62	Undet	Undet
GI.7 (Winchester)	RNA fragment	RIVM	17.54	Undet	Undet
GI.8	Faeces	REGA	22.76	Undet	27.32
GI.10 (Boxer)	RNA fragment	RIVM	19.34	Undet	Undet
GII.1 (Hawaii)	RNA fragment	RIVM	Undet	19.46	Undet
GII.2 (Melksham)	RNA fragment	RIVM	Undet	18.66	Undet
GII.2	Faeces	REGA	Undet	29.92	27.76
GII.3 (Toronto)	RNA fragment	RIVM	Undet	21.78	Undet
GII.4 (Grimsby)	RNA fragment	RIVM	Undet	18.26	Undet
GII.4	Vomit	REGA	Undet	28.95	27.83
GII.4	Faeces	REGA	Undet	22.90	27.82
GII.4	Faeces	REGA	Undet	21.63	28.78
GII.?	Faeces	IPH	Undet	28.92	27.79
GII.?	Faeces	IPH	Undet	26.30	27.41

GII.?	Faeces	IPH	Undet	33.57	27.89
GII.?	Faeces	IPH	Undet	25.72	27.37
GII.?	Faeces	IPH	Undet	26.28	27.61
GII.?	Faeces	IPH	Undet	27.05	27.58
GII.6 (Seacroft)	RNA fragment	RIVM	Undet	22.07	Undet
GII.7	Faeces	IPH	Undet	21.48	Undet
GII.10 (Erfurt)	RNA fragment	RIVM	Undet	18.49	Undet
GIIb (GGIIb)	RNA fragment	RIVM	Undet	19.05	Undet
GIIc (GGIIc)	RNA fragment	RIVM	Undet	19.21	Undet
GIV (Alphatron)	RNA fragment	RIVM	35.87	Undet	Undet
GIII (Bovine)	Faeces	Ulg[d]	Undet	Undet	Undet
GIII (Bovine)	Faeces	Ulg	Undet	Undet	Undet
GIII (Bovine)	Faeces	Ulg	Undet	Undet	Undet
GIII (Bovine)	Faeces	Ulg	Undet	Undet	Undet
Rotavirus	Faeces	RIVM	Undet	Undet	Undet
Astrovirus type 1	Faeces	RIVM	Undet	Undet	Undet
Astrovirus type 4	Faeces	RIVM	Undet	Undet	Undet
Sapovirus	Faeces	RIVM	Undet	Undet	Undet
Feline Calicivirus	Faeces	RIVM	Undet	Undet	Undet
Canine Calicivirus	Faeces	RIVM	Undet	Undet	Undet
Hepatitis A virus	Faeces	RIVM	Undet	Undet	Undet

[a]IPH: Belgian Scientific Institute of Public Health, [b]RIVM: Dutch National Institute for Public Health and the Environment, [c]REGA: Rega Institute for Medical Research, [d]Ulg: Liège University, [e] VTM: Viral transport medium.

In conclusion, the achievement of the first objective in the NORISK project involved the design of a multiplex real-time RT-PCR assay for simultaneous detection of human GI and GII NV in clinical samples, with the successful inclusion of MNV-1 as real-time PCR IAC. This multiplex real-time PCR assay can be used as a rapid method for detection of NV in environmental and food samples as well, although the robustness of this assay should be further examined for these sample categories. The latter was performed during the second objective of the NORISK project, in combination with the evaluation of two methods for virus extraction from soft red fruits and RTE foods.
For the collection clinical GI NV en GII NV samples, non-NV samples and NV-negative samples, contacts were made with the Rega Institute for Medical Research (Laboratory of Clinical and Epidemiological Virology, Department of Microbiology and Immunology), with the National Institute for Public Health and the Environment (RIVM; Centre for Infectious Disease Control, Laboratory for Infectious Diseases and Perinatal Screening), University of Liege (Ulg; Faculty of Veterinary Medicine) and the Scientific Institute of Public Health (WIV-ISP) . Additionally, the RIVM provided an opportunity to follow the full diagnostic procedure for investigation of clinical and environmental samples towards NV presence.

In the early phase of the development of the multiplex real-time PCR assay, contamination issues were encountered by frequent observation of positive no template

controls (NTCs). Investigation towards the source of the positive NTCs was believed to be necessary because of the need for reliable detection of 10 or less NV genomic copies per PCR reaction. A suspicion of well-to-well migration of positive control DNA (a short synthetic single stranded DNA (ssDNA) fragment) during real-time PCR runs was uttered as hypothetic cause of the positive NTCs. Results showed that evaporation of water occurred during real-time PCR runs regardless of the DNA type and the real-time PCR reaction plate seal type. It was also suggested that co-evaporation of DNA took place, with an apparent negative correlation between the size of the DNA type and the extent of this co-evaporation. Cloning of the short DNA fragments into commercial vectors and using these plasmids (pGI, pGII and p20.3) as positive controls has resolved the contamination problem and no consistent contamination problems have been observed further.

DEVELOPMENT OF METHODS FOR EXTRACTION AND CONCENTRATION OF NOROVIRUS (= SAMPLE PREPARATION) FROM A BROAD RANGE OF FOOD PRODUCTS

For the second objective, different approaches were selected according to the tested food category.

As proposed by Baert et al. (5), a single method for extraction of NV from all food matrices is most likely not possible and three different food categories could be considered according to the components within the food matrix. A first category of foods contains fats and proteins rather than carbohydrates and covers a broad range of food products. In this category, ready-to-eat (RTE) foods such as composite meals and deli sandwiches have been mostly implicated with NV food borne outbreaks. A second category of foods contains mostly carbohydrates and water, while proteins and fats are low abundant. This category consists mainly of fresh produce, of which raspberries and salad vegetables have been most frequently inflicted with NV food borne outbreaks. Bivalve molluscan shellfish were considered a third class, because of their ability to accumulate viral pathogens in the digestive tissue which allows development of virus detection methods specific for this food category. Examples of this food category often involved in NV food borne outbreaks are oysters and mussels.

For soft red fruits (selected as typical carbohydrate/water based food), an alkaline elution – PEG precipitation approach described by Baert et al. (5), which is very similar to the protocol described by the CEN/TC275/WG6/TAG4 working group, was thoroughly evaluated. This method included i) elution of the virus particles from the food surface using an alkalic buffer (pH 9.5) followed by ii) concentration of the eluted virus particles concentration. Polyethylene glycol (PEG, a protein binding molecule) was used for the latter purpose. Subsequently, the precipitated virus particles were subjected to a

chloroform:butanol purification to remove inhibitory substances. Finally, RNA was purified from the precipitated virus particles using the manual QIAamp RNeasy minikit (Qiagen).

For RTE foods, a careful evaluation was performed of a promising protocol for RTE foods consisting of a long (TriConc) and short (TriShort) variant (5). Both protocols involved treatment of the food product with a guanidinium isothiocyanate (GITC) and phenol-based reagent to extract RNA followed by an eventual purification/precipitation of the extracted RNA. The combination of GITC and phenol allows the maintaining of the integrity of the extracted RNA while disrupting/lysing cells and solubilizing cell components (19, 20). For the TriShort protocol, RNA was directly purified from the nucleic acid extract, while an additional organic solvent purification/concentration step was performed in the TriConc protocol.

Finally, the extracted RNA was purified using either the manual QIAamp RNeasy minikit (Qiagen) or the automated NucliSens EasyMAG (BioMérieux). Throughout this report, both methods will be referred to as "manual RNA purification" and "automated RNA purification", respectively.

To achieve this objective of the NORISK project, protocols for extraction and concentration of NV (= sample preparation) from fresh produce and ready-to-eat foods were evaluated towards robustness and sensitivity by analyzing the influence of (1) the NV inoculum level and (2) different food types on the recovery of NV from these foods. The recovery of GI and GII NV and MNV-1 from the food samples was both qualitatively and quantitatively analyzed. Firstly, qualitative analysis of the recovery of the GI and GII NV and MNV-1 inocula from food samples was defined as the "recovery success rate". This recovery success rate was calculated as "the number of PCR reactions showing successful recovery of GI/GII NV or MNV-1" per "number of PCR reactions performed". Secondly, quantitative analysis of the recovery of GI and GII NV or MNV-1 from individual food samples was defined as "recovery efficiency". This recovery efficiency (%) was calculated per individual sample as "the mean recovered number of GI and GII NV or MNV-1 genomic copies" per "mean inoculated number of GI/GII NV or MNV-1 genomic copies".

First of all, sensitivities of both protocols were analyzed on penne salad and raspberry crumb, selected as typical RTE food and soft red fruit product, respectively. For this purpose, high ($\sim 10^6$-10^7 NV genomic copies / 10 g) and low ($\sim 10^4$-10^5 NV genomic copies / 10 g) levels of GI and GII NV were inoculated on these food products. A high level MNV-1 inoculum was added to the samples as process control (MNV-1 PC).

Results for both protocols showed that the inoculum level of GI and GII NV had a significant influence on its recovery for both protocols.

For the protocol for RTE foods, the high level GI and GII NV inocula could be recovered from penne salad samples (10 g) in at least 4 out of 6 PCRs, while the low level GI and

GII NV inocula could be recovered from this food product in maximally 3 out 6 PCRs (Table 3). For the high inoculum levels, the TriShort and TriConc protocols resulted in mean recovery efficiencies of >1 % and 0.1 to 10 %, respectively. The MNV-1 PC was successfully evaluated as it could be recovered by both protocol variants in all but a single case and with similar recovery efficiencies compared to the high level NV inocula. An overview of the results is shown in Table III.

Table III. Overview of the results obtained when evaluating the influence of the virus inoculum level on the recovery of GI NV, GII NV and MNV-1 from penne salads using the protocol for RTE foods.

	TriShort				
	GI NoV[c]		GII NoV[c]		MNV-1 PC[c]
Recovery efficiency[a]	1.40×10^6	4.02×10^3	5.61×10^5	4.91×10^4	1.41×10^6
> 10 %	1/12	1/12	3/12		14/32
1 % - 10 %	8/12		5/12		17/32
0.1 % - 1 %	2/12				
No recovery	1/12	11/12	4/12	12/12	1/32
Mean recovery ± stdev[b]	3.8 % ± 3.9 %	49.0 %	11.5 ± 7.3 %	/	11.8 % ± 10.1 %
	TriConc				
	GI NoV[c]		GII NoV[c]		MNV-1[c,d]
Recovery efficiency[a]	1.40×10^6 gen cop /10g	4.02×10^3 gen cop /10g	5.61×10^5 gen cop /10g	4.91×10^4 gen cop /10g	1.41×10^6 gen cop /10g
> 10 %		2/12	2/12		6/32
1 % - 10 %	2/12	1/12	3/12	2/12	15/32
0.1 % - 1 %	10/12		6/12		11/32
No recovery		9/12	1/12	10/12	
Mean recovery ± stdev[b]	1.0 % ± 1.5 %	12.0 % ± 8.8 %	3.3 % ± 5.6 %	8.4 % ± 1.3 %	4.2 % ± 4.8 %

[a] ("mean recovered number of GI/GII NV or MNV-1 genomic copies" / "mean inoculated number of GI/GII NV or MNV-1 genomic copies") × 100 %.

[b] stdev: standard deviation

[c] inoculum level expressed as genomic copies / 10 g food sample.

[d] MNV-1 added to the penne salad samples was prepared as a virus lysate as described by (7).

A similar protocol combining the use of TRIzol® Reagent with an additional RNA purification step has been able to recover 104 RT-PCR units of NV per 40 g of ham and turkey meat with a ~1 % efficiency, while no recovery was possible in roast beef meat (111). In another study, 2.5×10^5 TCID50 of canine Calicivirus (CaCV) were inoculated on lettuce and whipped cream and could be recovered with recoveries ranging between

1 % and 10 %, using a NaCl-PEG precipitation step in addition to a TRIzol®-based protocol for RTE foods (108). The latter study also demonstrated a 1 % recovery efficiency of the CaCV inoculum in macaroni, a food product similar to the penne salad. Recovery success rates of the high level NV inocula were significantly higher compared to the low level NV inocula. In agreement with the presented results, Cheong et al. (17) demonstrated that the recovery of NV in lettuce was more successful at high concentrations, both in large and small volumes of tested food samples.

For the protocol for soft red fruits, the high and low level GI and GII NV inocula could be recovered from raspberry crumb samples (10 g) in 20 to 23 out of 24 PCR reactions (Table IV). However, the high level inocula could be recovered more efficiently (12.28 ± 1.09 % to 28.44 ± 3.09 %) compared to the low level NV inocula (2.93 ± 0.93 % to 11.79 ± 7.30 %). The MNV-1 PC was successfully evaluated as it could be recovered in all cases and with similar recovery efficiencies compared to the NV inocula. The MNV-1 RTC and MNV-1 IAC were detected without matrix at Ct values of 23.61 and 30.34, respectively and were in all samples detected at Ct values of 24.06 ± 0.32 and 30.11 ± 0.41, indicating no inhibition of reverse transcription and real-time PCR reactions. An overview of the results is shown in Table IV.

Table IV. Influence of NV inoculum level on NV extraction efficiencies from 10 grams of artificially contaminated deepfrozen raspberry crumb samples. Every inoculation (combination) was duplicated.

Inoculum	GI NoV Inoculum level (genomic copies/10g)	GII NoV Inoculum level (genomic copies/10g)	Recovery efficiency[a] GI NoV ± stdev (success rate)[b]	Recovery efficiency[a] GII NoV ± stdev (success rate)[b]	Recovery efficiency[a] MNV-1 PC ± stdev (success rate)[b]
GI NoV	1.47×10^7	/[c]	28.44 ± 3.09 % (4/8)	Negative	12.79 ± 2.10 % (4/4)
	1.95×10^5	/	6.41 ± 4.64 % (8/8)	Negative	14.34 ± 1.94 % (4/4)
GII NoV	/	7.09×10^7	Negative	12.79 ± 2.93 % (8/8)	12.40 ± 0.42 % (4/4)
	/	2.32×10^6	Negative	5.70 ± 1.47 % (8/8)	13.63 ± 2.04 % (4/4)
GI + GII NoV	1.47×10^7	7.09×10^7	22.05 ± 8.31 % (8/8)	15.18 ± 5.39 % (8/8)	15.99 ± 5.84 % (4/4)
	1.47×10^7	2.32×10^6	19.26 ± 4.64 % (8/8)	2.93 ± 0.93 % (8/8)	14.18 ± 6.32 % (4/4)
	1.95×10^5	7.09×10^7	9.71 % (1/8)	12.28 ± 1.09 % (7/8)	20.49 ± 1.29 % (4/4)
	1.95×10^5	2.32×10^6	11.79 ± 7.30 % (5/8)	7.00 ± 1.38 % (5/8)	19.61 ± 1.71 % (4/4)
Negative control	/	/	Negative	Negative	15.69 ± 7.06 % (4/4)

[a] ((Mean number GI/GII NV or MNV-1 genomic copies recovered from 10g of inoculated fruit sample)/(Number GI/GII NV or MNV-1 genomic copies inoculated on 10g of fruit sample)) × 100 %

[b] # positive real-time PCR reactions / # performed real-time PCR reactions

[c] Not added

A similar NV elution-concentration protocol designed by Butot et al. (13) recovered 2160 RT-PCRU of GI.4 NV per 60 g of food product with efficiencies of 1.7%, 2.6%, 17.9% and 19.6% in fresh strawberries, frozen raspberries, frozen blueberries and fresh

raspberries, respectively. In a recent study, GI and GII NV were extracted from artificially contaminated strawberries by combining a similar NV elution-concentration method with an immunomagnetic separation technique (98). In the latter study, 4×10^3 – 10^4 GI and GII NV RT-PCRU could be recovered with efficiencies of 29.50% and 14.14%, respectively. A recent study comparing different aspects of the NV elution - PEG concentration method (using conventional RT-PCR) showed that 85% recovery of 4×10^4 GII.4 NV RT-PCRU from fresh strawberries was possible when combining a 3% beef extraction buffer as elution buffer with 8% (w/v) PEG8000 precipitation (52). Finally, Cheong et al. (17) obtained 3.9% to 50% recoveries when extracting 4.8×10^0 – 10^3 GII NV RT-PCRU from 5 g of strawberries by comparing different elution buffers in a similar elution-concentration detection protocol.

Secondly, the robustness of both protocols was evaluated. For this purpose, the influence of the food type on the recovery of NV was investigated by inoculating different foods with low levels of NV ($\sim 10^4$ NV genomic copies / 10 g).
For the direct RNA protocol, 10 g samples of three categories of RTE foods (4 deli sandwiches, 4 soups and 14 composite meals) were inoculated with a combined low level GI NV and GII NV and high level MNV-1 inoculum (the latter serving as process control). Simultaneously, the effect of two RNA purification methods (manual RNeasy minikit (Qiagen) and automated NucliSens EasyMAG (BioMérieux)) on the recovery of NV from these RTE foods was examined. An overview of the results is shown in Table V. For soups, both the automated RNA purification and TriConc protocol provided significant better results compared to the manual RNA purification and TriShort protocol, respectively. Automated RNA purification generated in the composite meals significant better results compared to the manual RNA purification, while the virus extraction method (TriShort or TriConc) did not affect the results significantly. Recovery of the GI and GII NV inocula in deli sandwiches was difficult, regardless of the used virus extraction protocol or RNA purification method. The high level MNV-1 inoculum could be detected in all PCR reactions, except in one case (Table V).
The difficult recovery of the inocula from deli sandwiches can most likely be explained by the absorbing properties of the food matrix (lowering the recovered volume of nucleic acid extract). However, a 1 % recovery of a CaCV inoculum from white bread has been observed before using a similar protocol, in combination with a NaCl-PEG precipitation (108). Nevertheless, an investigation of NV outbreaks related to the consumption of deli sandwiches showed that in most cases, NV could not be detected in these RTE food products (24, 26, 27, 30, 100). Further research might therefore in particular be recommended to improve the recovery of low level NV inocula from this food type.

Table V. Overview of the results obtained when evaluating the influence of RTE food type (4 deli sandwiches, 4 soups and 14 composite meals) on virus recovery.

Inoculum	Inoculum level[a]	RNA isolation[b]	Recovery success rate					
			Deli sandwiches (n=4)		Soups (n=4)		Composite meals (n=14)	
			Tri short	Tri conc	Tri short	Tri conc	Tri short	Tri conc
GI NoV	1.28×10^4	Manual	1/8 [c]	2/8	1/8	2/8	7/28	5/28
		Automated	2/8	0/8	4/8	7/8	11/28	18/28
GII NoV	5.81×10^4	Manual	1/8	3/8	1/8	3/8	8/28	4/28
		Automated	1/8	1/8	5/8	7/8	12/28	8/28
MNV-1	1.35×10^7	Manual	8/8	8/8	8/8	8/8	28/28	28/28
		Automated	8/8	7/8	8/8	8/8	28/28	28/28

[a] expressed as NV genomic copies per 10g food. Every sample was inoculated with the combined GI NV, GII NV and MNV-1 inoculum.

[b] manual: RNeasy minikit (Qiagen), automated: NucliSens EasyMAG (BioMérieux)

[c] # positive real-time PCR reactions / # performed real-time PCR reactions

For the robustness analysis of the protocol for soft red fruits, 10 g samples of three soft red fruits (deep frozen forest fruit mix, fresh raspberries or strawberry puree) were inoculated with low level GI NV and GII NV and high level MNV-1 inocula (the latter inoculum serving as process control). In contrast to the direct RNA extraction method, only a single RNA purification method was used. An overview of the results is shown in Table VI.

Results showed a significant influence of the soft red fruit product type on the recovery efficiency of NV GI and MNV-1, while no significant differences could be shown for GII NV.

In general, the recovery of NV was more efficient and successful from the strawberry puree compared to a frozen forest fruit mix and fresh raspberries.

The effect of different food matrices on the quantification of NV in food products has only been investigated by a limited number of authors. A recent study combining carbohydrate-coated magnetic beads with conventional RT-PCR showed that the recovery of NV from lettuce and green onions had a higher success rate compared to the recovery of NV from fresh strawberries (83). Results obtained in the current study were in contrast to a very similar extraction method developed by Dubois et al. (32). The latter study showed a tenfold less efficient recovery of the NV elution-concentration method when tested on mashed strawberries compared to frozen raspberries and fresh strawberries.

Table VI. Influence of soft red fruit type on NV extraction efficiencies from artificially contaminated soft red fruit products. Inoculation(s) (combinations) were not duplicated.

Food type	GI NoV Inoculum level (genomic copies/10g)	GII NoV Inoculum level (genomic copies/10g)	Recovery efficiency[a] GI NoV ± stdev (recovery success rate)[b]	Recovery efficiency[a] GII NoV ± stdev (recovery success rate)[b]	Recovery efficiency[a] MNV-1 PC (recovery success rate)[b]
Deepfrozen	3.99×10^4	/[c]	7.42 ± 2.65 % (2/4)		23.65% (2/2)
forest fruit mix	/	9.63×10^4		Negative (0/4)	7.78% (2/2)
	3.99×10^4	9.63×10^4	13.47 ± 7.72 % (4/4)	20.68 ± 18.27 % (4/4)	28.78% (2/2)
	/	/	Negative	Negative	32.78% (2/2)
Fresh	3.99×10^4	/	21.50 ± 6.74 % (4/4)		8.29% (2/2)
raspberries	/	9.63×10^4		35.20 ± 31.54 % (2/4)	25.76% (2/2)
	3.99×10^4	9.63×10^4	Negative (0/4)	Negative (0/4)	21.10% (2/2)
	/	/	Negative	Negative	12.87% (2/2)
Fresh	3.99×10^4	/	51.13 ± 38.24 % (4/4)		52.05% (2/2)
strawberry	/	9.63×10^4		47.72 ± 25.43 % (4/4)	39.64% (2/2)
puree	3.99×10^4	9.63×10^4	61.06 ± 40.11 % (4/4)	25.26 ± 19.08 % (3/4)	75.65% (2/2)
	/	/	Negative	Negative	42.23% (2/2)

[a] ((Mean number GI/GII NV or MNV-1 genomic copies recovered from 10g of inoculated fruit sample)/(Number GI/GII NV or MNV-1 genomic copies inoculated on 10g of fruit sample)) × 100 %

[b] # positive real-time PCR reactions / # performed real-time PCR reactions

[c] Not added

In conclusion, the achievement of the second objective involved (1) the evaluation of a direct RNA extraction protocol for detection of NV in RTE foods and (2) the evaluation of an elution-precipitation protocol for detection of NV in soft red fruits.

The direct RNA extraction protocol (TriShort and TriConc protocol variants) was, in combination with the multiplex real-time RT-PCR assay described in in the first objective of the NORISK project, capable of reliably detecting high concentrations (10^5 to 10^6 genomic copies / 10 g food sample) of NV in RTE foods, while detection of lower NV levels ($\sim 10^4$ genomic copies / 10 g food sample) was more difficult. However, as outbreaks can be caused by only 10-100 NV particles, current detection methods may still lack the necessary sensitivity to detect these very low concentrated viral agents in the suspected foods. This may explain why reported viral foodborne outbreaks related to RTE foods are based on epidemiological data rather than on actual detection of the viral particles in the food (90, 91). Detection of low NV levels was also influenced by the food type. Nevertheless, aided by a few modifications towards difficult matrices such as deli sandwiches, this method could be used for analysis of RTE foods implicated for NV presence. Moreover, both protocols did not generate significantly different results, except for soups. Therefore, the more laborious TriConc protocol could function as an additional virus extraction when samples tested negative using the TriShort protocol. However, a food sample testing negative for NV does not automatically mean total

absence of NV, due to the observed limitations of the presented method (in particular with deli sandwiches).

The protocol for soft red fruits was, in combination with the multiplex real-time RT-PCR assay described in the first objective of the NORISK project, capable of reliably detecting high (10^5 to 10^6 NV genomic copies / 10 g food sample) and low concentrations (10^4 NV genomic copies / 10 g food sample) of NV in soft red fruits. Detection of low concentrated NV levels was also influenced by the food type, as NV detection was generally more successful in fresh strawberry puree compared to a frozen forest fruit mix or fresh raspberries. Nevertheless, this method could be used for analysis of RTE foods implicated for NV presence. This detection methodology was applied for achievement of the fourth objective of the NORISK project by screening of 75 fruit samples for NV presence.

Finally, the murine norovirus-1 was successfully included as full detection procedure process control, reverse transcription control and real-time PCR internal amplification control in the protocol for soft red fruits. In the protocol for RTE foods, MNV-1 was only evaluated as PC.

During evaluation of the protocol for RTE foods, thesis student Ann De Keuckelaere was trained to perform the multiplex real-time RT-PCR assay as well as the direct RNA extraction protocol.

DEVELOPMENT AND IMPLEMENTATION OF A PROTOCOL WITH ESTABLISHMENT OF APPROPRIATE CONTROLS FOR RAPID SCREENING OF FOODS

For the third objective, the molecular methods described in the first objective were combined with the virus extraction protocols described in objective 2. This leads to protocols for detection of NV in RTE foods (Fig 3A), soft red fruits (Fig 3B) and shellfish (Fig 3C).

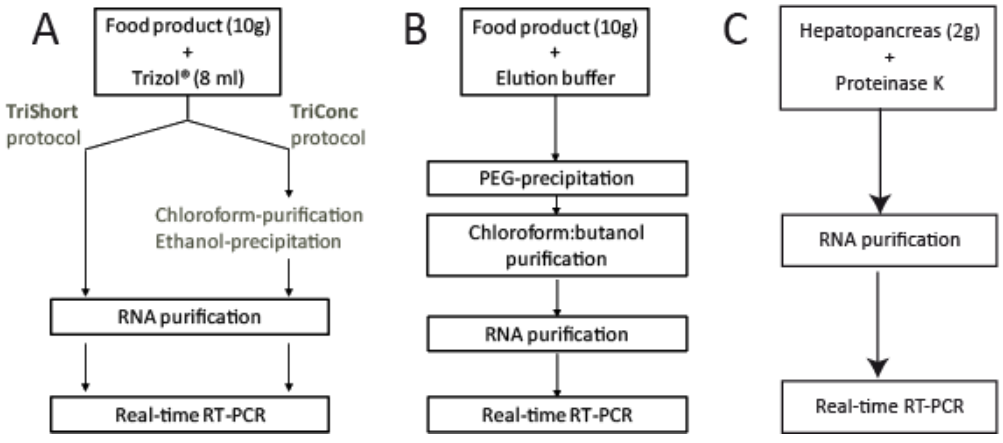


Fig. 3. Overview of methods for detection of NV in soft red fruits (A), ready-to-eat foods (B) and shellfish (C)

For the methodology for detection of NV in soft red fruits and RTE foods, MNV-1 was used to control the entire virus detection protocol (process control; MNV-1 PC), the reverse transcription reaction (reverse transcription control; MNV-1 RTC) and the real-time PCR reaction (internal amplification control; MNV-1 IAC). For the MNV-1 PC, a known amount of MNV-1 virus particles was added to the food sample and was thus a estimation for the efficiency whereby NV could be detected in the tested food sample. The MNV-1 RTC and IAC consisted of the addition of (a known amount of) genomic MNV-1 RNA to the reverse transcription reaction mix and the addition of (a known amount of) plasmid containing a full length cDNA copy of the MNV-1 genome to the real-time PCR reaction, respectively. The latter controls were used to detect possible inhibition of the real-time RT-PCR.

The use of these controls was combined to obtain more specific information on which steps of the NV detection methods were more susceptible to inhibition and which steps might reduce the overall sensitivity. The inclusion of a process control and an internal amplification control has been suggested for detection of foodborne viruses by the Belgian Superior Health Council (2010), while ISO 22174:2005 required the use of an adequate PC and IAC for detection of food borne pathogens by PCR. Additionally, the reverse transcription reaction has been known to be prone to inhibition when detecting viral agents in shellfish (64, 79, 110), water (59) and other foods (66) and therefore needs to be controlled.

The use of MNV-1 as PC was chosen as control reagent besides other successfully tested viruses because of the closer genomic relationship to human infective NV. Other successfully used PCs for detection of viral pathogens in food include the feline calicivirus (FCV), F-RNA bacteriophage MS2, and a genetically modified mengovirus (vMC_0). Consequently, MNV-1 was also used for the RTC and IAC.

The use of MNV-1 as PC was successful for detection of NV in several soft red fruits and RTE foods, but a sufficiently high concentration (10^6 genomic copies/10 g food product) is needed to assure detection and reliable estimation of its recovery efficiency. Both in soft red fruits and RTE foods, recovery efficiency of the MNV-1 PC was in most cases similar to recovery of the GI/GII NV, if inoculated in same levels (10^6 genomic copies/10g). Regarding the evaluation of MNV-1 as RTC and IAC, results obtained during evaluation of the virus extraction protocols (objective 2) suggest that the reverse transcription reaction was more prone to inhibition compared to the real-time PCR reaction when working in foods.

Consequently, a shortened NV detection strategy for soft red fruits and RTE foods could be suggested (Fig. 4). In this strategy, the MNV-1 IAC is left out, while the MNV-1 RTC and MNV-1 PC are used in 2 subsamples to (1) control the detection efficiency and (2) detect possible inhibition of the real-time RT-PCR . To avoid competition between the

individual reactions within the multiplex assay, the concentration of the MNV-1 PC should be limited to 10^6 genomic copies / 10 g food.

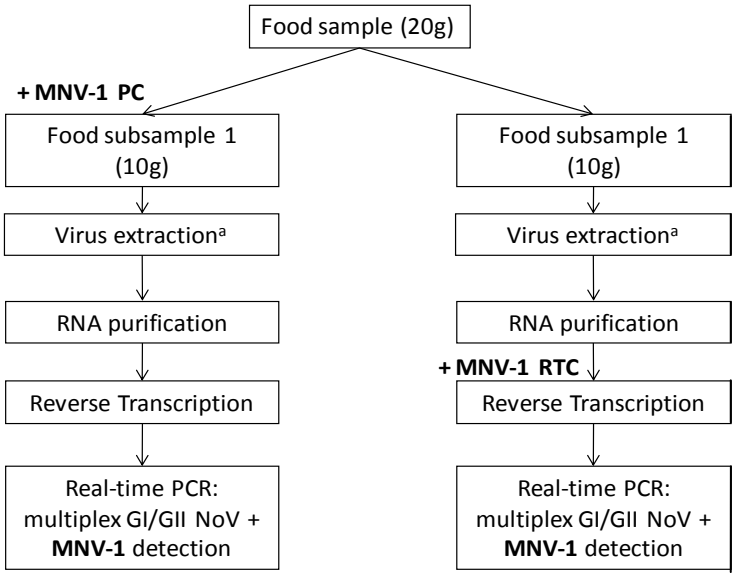


Fig. 4. Proposed detection strategy including MNV-1 as process control (MNV-1 PC) and reverse transcription control (MNV-1 RTC). [a] Virus extraction: the evaluated direct RNA extraction method (TriShort or TriConc variant) for RTE foods or the evaluated elution-precipitation protocol for soft red fruits.

ELUCIDATION OF TRANSMISSION ROUTES OF NOROVIRUSES IN FOOD

For the elucidation of transmission routes of NV in foods (as part of the **fourth objective)**, a total of 75 fruit products (raspberries, strawberries, cherry tomatoes and fruit salads) were screened for NV presence. Additionally, the presence of three bacterial pathogens (*E. coli* 0157:H7, *Salmonella*, and *Listeria spp./monocytogenes*) and enumerations of *Enterobacteriaceae* and *E. coli* were analyzed in all tested fruit samples to investigate a possible link between bacterial presence and the detection NV. The use of MNV-1 as PC, RTC and IAC was evaluated during this screening. Additionally, the fruit samples were screened for bacterial pathogens and bacterial hygiene indicators.
For raspberries, two lots (originating from Serbia and Poland) containing five samples originating from five different farmers were tested. For cherry tomatoes and strawberries, respectively three and two lots (originating from Spain) containing each ten samples were analyzed. Finally, fifteen mixed fruit salads (prepared in Belgium) were examined. All samples were friendly provided by local manufacturers and distributors. For this objective, the NV detection methodology (including the use of MNV-1 as control reagent) for soft red fruits described in the third objective was applied. An overview of the NV detection strategy is shown in Fig. 5.

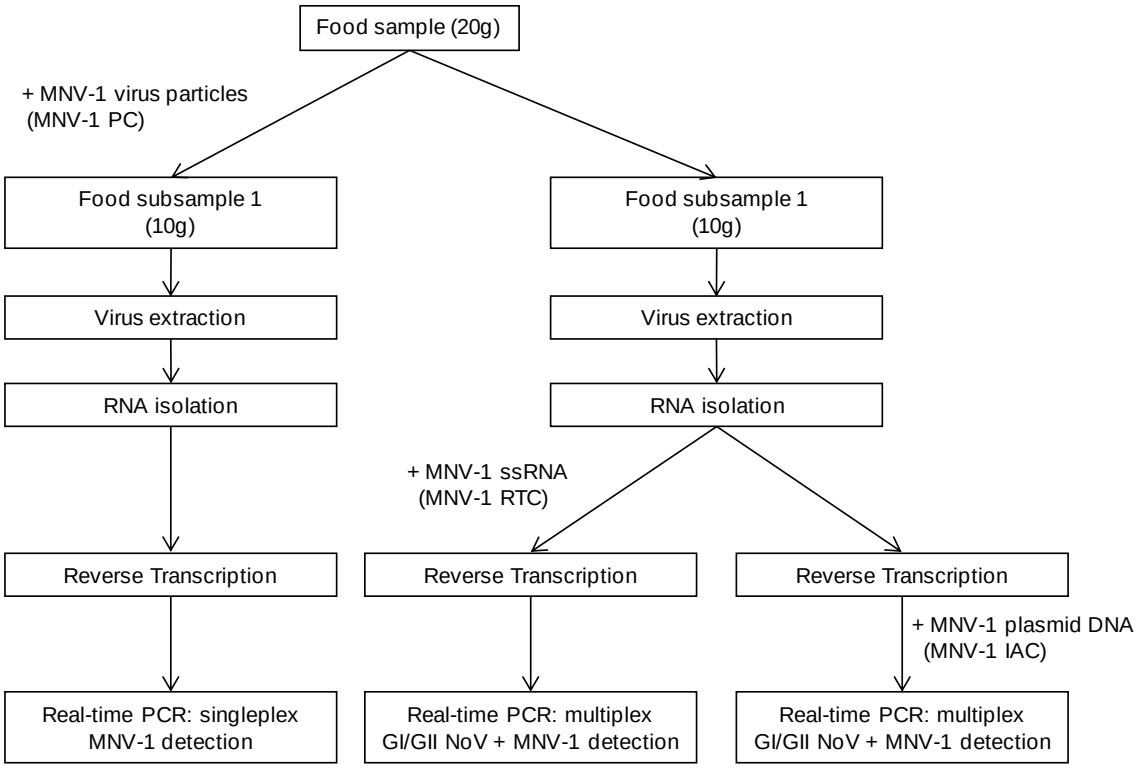


Fig. 5. Overview of the strategy used for detection of NV in soft red fruit products.

In total, 2 singleplex real-time PCR reactions for detection of the MNV-1 PC were performed in the first 10 g food subsample (inoculated with MNV-1 PC). In the second 10 g food subsample (inoculated with GI and/or GII NV), 4 multiplex real-time PCR reactions were executed for the detection of GI and GII NV (2 reactions with the MNV-1 RTC and 2 reactions with the MNV-1 IAC).

The recovery of the MNV-1 PC/RTC/IAC was both quantitatively and qualitatively analyzed. Quantitative analysis was performed by comparing the mean recovered number of MNV-1 PC/RTC/IAC genomic/RNA/plasmid copies with the mean inoculated number of MNV-1 PC/RTC/IAC genomic/RNA/plasmid copies.

Qualitative analysis of the recovery of the MNV-1 PC/RTC/IAC was calculated by comparison of the number of positive MNV-1 real-time PCR signals to the number of performed MNV-1 real-time PCR reactions, per control type. The quantitative and qualitative analyses were expressed respectively as the "recovery efficiency" and "recovery success rate".

Regarding the evaluation of MNV-1 as control reagent, results showed that successful recovery of the MNV-1 PC was dependent of the fruit type tested: while recovery success rates of 8/10 and 19/20 were noticed in respectively raspberries and strawberries, recovery success rates of 2/30 and 2/15 were noticed in cherry tomatoes and mixed fruit salads, respectively. However, it was expected that a higher concentrated (10^6 MNV-1 genomic copies/10g fruit sample) MNV-1 PC would have

resulted in a higher recovery success rate of this PC, as a concentration of only ~10^4 MNV-1 genomic copies/10g was used. Quantitative analysis showed that the mean recovery efficiency of the successfully recovered MNV-1 PCs was similar in all tested fruit types as mean recovery efficiencies ranged between 8.38 ± 1.18 % and 12.94 ± 9.33 % (Table VII). The MNV-1 RTC and IAC could be detected in all but a single raspberry sample. The MNV-1 RTC was recovered with mean efficiencies ranging between 46.17 ± 17.70 % and 63.81 ± 15.72 %, while the MNV-1 IAC was recovered with mean recovery efficiencies ranging between 100.93 ± 9.55 % and 119.32 ± 28.32 %. The rather low recovery of the MNV-1 RTC indicated limited inhibition of the reverse transcription step in some samples. In combination with the results obtained during evaluation of the virus extraction protocols (objective 2) and the fact that the MNV-1 IAC was recovered with high efficiencies in all but a single raspberry sample, a shortened NV strategy has been proposed as shown in Fig 5.

Table VII. Quantitative and qualitative analysis of the performance of MNV-1 as process control (MNV-1 PC), reverse transcription control (MNV-1 RTC) and real-time PCR internal amplification control (MNV-1 IAC).

Fruit type	N	MNV-1 PC		MNV-1 RTC	MNV-1 IAC
		Mean recovery efficiency[a] ± stdev (Recovery success rate)[b]	Recovery efficiency range	Recovery efficiency[a] ± stdev (Recovery success rate)[b]	Recovery efficiency[a] ± stdev (Recovery success rate)[b]
Raspberries	10	12.94 ± 9.33 % (8/10)	2.79 % - 27.27 %	46.17 ± 17.70 % (9/10)	100.93 ± 9.55 % (9/10)
Cherry tomatoes	30	11.17 ± 8.22 % (2/30)	2.57 % - 19.78 %	56.13 ± 12.31 % (30/30)	117.76 ± 9.22 % (30/30)
Strawberries	20	12.80 ± 5.60 % (19/20)	5.35 % - 19.68 %	46.54 ± 29.75 % (20/20)	114.27 ± 19.03 % (20/20)
Mixed fruit salad	15	8.39 ± 1.18 % (2/15)	7.56 % - 9.23 %	63.81 ± 15.72 % (15/15)	119.32 ± 28.32 % (15/15)
Total	75	11.32 ± 6.08 % (31/75)	2.57 % - 27.27 %	53.16 ± 18.87 % (74/75)	114.75 ± 15.70 % (74/75)

[a] Recovery efficiency: (Mean concentration MNV-1 genomic/RNA/plasmid copies inoculated) / (mean concentration MNV-1 genomic/RNA/plasmid copies recovered)) × 100 %.

[b] Recovery success rate: (# samples per fruit type with successful recovery of the MNV-1 PC/RTC/IAC) / (# samples per fruit type).

Regarding the screening of these fruit sample for NV presence, results showed that 18 samples tested positive for GI and/or GII NV genomic material despite a good bacteriological quality (data not shown). The level of detected NV genomic copies ranged between 2.5 and 5.0 logs per 10 grams of fruit sample. NV GI and/or GII were found in 4/9, 7/30, 6/20 and 1/15 of the tested raspberries, cherry tomatoes, strawberries and fruit salad samples, respectively. It should be noted that MNV-1 PC, RTC and IAC tested negative in a single raspberry sample, hence no conclusions could be drawn from this sample regarding NV presence. An overview of all NV positive samples is shown in Table VIII. Since the observed Ct values of the positive samples ranged between 37 and

42, it is important to mention that all negative template controls (NTCs) were negative, thus excluding positive real-time PCR signals due to PCR contamination. Contamination-preventing measures such as the use of dedicated environmental conditions (separate working areas, UV and hypochlorite decontamination, dedicated pipettes) and the use of uracil DNA-glycosilase (UNG) containing real-time PCR mastermixes were respected at all time.

Table VIII. Overview NV genomic presence on tested fruit samples.

	Sample name	GI NoV presence[a] (Ct value(s))	GII NoV presence[a] (Ct value(s))	MNV-1 PC	MNV-1 RTC	MNV-1 IAC
Raspberries (n=10)	RB P03 20090421	nd[b]	3.05 (39.98)	15.35%	66.29%	104.58%
	RB P05 20090421	2.61 (40.81)	3.70 (37.66)	4.01%	54.56%	104.58%
	RB S01 20090421	2.45 (41.24; 41.47)	3.60 (38.03)	nr[c]	36.71%	93.57%
	RB S02 20090421	3.21 (38.74)	nd	8.73%	27.99%	81.14%
Cherry tomatoes (n=30)	CT 07 20090423	nd	3.91 (38.66)	nr	60.88%	114.08%
	CT 09 20090423	4.11 (40.67)	nd	nr	75.92%	123.20%
	CT 10 20090423	4.33 (39.98)	nd	nr	47.22%	126.12%
	CT 01 20090429	4.08 (38.54; 38.30)	nd	nr	39.35%	113.32%
	CT 04 20090429	nd	4.19 (41.31)	nr	59.22%	111.47%
	CT 06 20090429	4.07 (37.39; 39.53)	5.04 (38.38)	nr	62.84%	132.30%
	CT 03 20090513	4.38 (39.38)	4.67 (37.15)	16.98%	62.38%	122.60%
Strawberries (n=20)	SB 01 20090506	4.10 (39.27)	3.28 (39.75)	9.02%	101.61%	122.96%
	SB 04 20090506	nd	3.05 (40.58)	8.09%	66.44%	132.94%
	SB 06 20090506	nd	3.77 (38.01)	12.70%	58.16%	123.37%
	SB 03 20090520	2.29 (41.97)	nd	nr	21.27%	100.42%
	SB 04 20090520	2.86 (40.02)	nd	13.88%	12.45%	93.90%
	SB 07 20090520	3.40 (38.20)	nd	12.54%	58.79%	108.85%
Fruit salads (n=15)	FS 01 20090504	nd	4.64 (40.92; 40.19)	7.56%	75.67%	92.92%

[a] # of detected genomic NV copies per 10 g fruit product sample are expressed in log scale. [b] nd: not detected. [c] nr: not recovered

Due to the high number of real-time PCR positive results, confirmation of the results was attempted by subjecting NV positive cDNA preparations, RNA preparations and virus extracts to three conventional RT-PCR assays used for genotyping NV. Primer sets described by Kojima et al.(53), Vennema et al. (126) and Vinje et al. (130) targeting respectively genotyping regions A, C and D were applied. However, no NV sequences could be obtained despite intense efforts. It has been shown that real-time RT-PCR is 10^2 up to 10^4 fold more sensitive compared to conventional RT-PCR (10, 96), which may explain the failed confirmation since only 1 to 20 NV genomic copies were detected by real-time RT-PCR in the cDNA of most positive samples. It should be noted that the real-time PCR assay performed in the current study used primers and hydrolysis probes recommended by the CEN/TC275/WG6/TAG4 working group, which does not suggest confirmation as use of the hydrolysis probes should ensure the specific detection of NV genomic material.

Only a limited number of authors have investigated presence of enteric viruses on fruit products not related to food borne outbreaks. Recently, Mattison et al. (72) found NV presence in 148 out of 275 tested packaged leafy greens. Similar to our results, confirmation was also difficult although sequencing confirmed NV presence in 16 out of 148 positive samples. A similar study examining the presence of GI and GII NV, adenoviruses (AdV), enteroviruses (EV) and rotaviruses (RV) in irrigated vegetables was performed by (18). However, only 2 samples (lettuce and chicory) out of 30 tested positive for AdV, while a single spinach sample contained AdV as well as NV.

Shellfish As the amplification of NV genomes in positive shellfish samples requires nested RT-PCR to be detectable and actual facilities do not allow these kinds of manipulations due to a high risk of cross contaminations, genotyping was based upon the results of the qRT-PCR as described by the CEN protocol. A total of 285 bivalve mollusk samples (238 mussels, 44 oysters, 3 bittersweets) have been analyzed since the start of the project.

Table IX: Results of NV detection in shellfish during the period of 2007 – 2009

Matrices	Samples analyzed	Positives for NV	GI	GII	GI + GII
Mussels	238	35	9	32	6
Oysters	44	5	1	4	-
Bittersweets	3	1	-	1	-
Total	285	41	10	37	6

Fourty-one out of 285 (10%) shellfish samples were tested positive for GI and/or GII NV (Table IX). The majority (90%) of the positive samples were positive for GII although 6

samples were positive for both GI and GII. Mussels and oysters were similarly contaminated by NV with 14.7 and 11.4 % of positive samples respectively. This result indicates that matrix does not seem to have an effect on contamination by NV.
One out of 10 shellfish samples tested were positive for NV GI and/or GII. The high prevalence of NV in retail shellfish in Belgium is similar to what is described in studies from other European countries. An important question is if these positive shellfish constitute a risk for public health through consumption. Our results indicate that mussels and oysters seem to be equally exposed to NV contamination and could both cause illness. Unfortunately no outbreak reported could be linked to the consumption of these foodstuffs.
In the literature positive sequencing results were obtained from shellfish with a high level of contamination due to environmental contamination accidents responsible for large outbreaks linked to the consumption of these bivalves. Sequences could also be obtained from positive human stool samples where bivalves could be identified as the cause of the infection. Unfortunately, we did not encounter either of these two situations throughout our project.

Work package 2: Virus evolution

DETECTION AND GENOTYPING

Animal noroviruses
For the Belgian perspective, diarrheic faecal samples from domestic animals were screened for NV between 2007 and 2008. NV sequences were detected in cattle and in pigs whereas all other domestic animal samples (poultry, sheep, equine, cats and dogs) were found negative (table X). Indeed, genotyping of animal NVs was limited to bovine and porcine species as NV screening of the animal faeces from the stool bank realized in phase 1 did not enable the detection of NVs in other animal species.

Table X: Norovirus screening in animal feces samples (N/A: not applicable)

Species	Primer pairs	Positive sequences (calicivirus)	Samples analyzed	Blast result for amplicon (number)
Cattle	JV12/13	7	433	Bovine norovirus (38)
	CBECu F/R	34		
	AMG1	33		
	BEC	2	43	
Equine	JV12/13	0	101	Calicivirus-like sequences (2)
	P289/290	2		

Porcine	JV12/13	0	43	Porcine norovirus (2) Porcine sapovirus (5)
	P289/290	5		
	AMPO F/R	2		
Ovine	JV12/13	0	7	N/A
	P289/290	0		
	CBECu F/R	0		
Caprine	JV12/13	0	2	N/A
	CBECu F/R	0		
	P289/290	0		
Canine	JV12/13	0	60	Feline calicivirus (5) Sapovirus-like sequences (2)
	P289/290	7		
Feline	JV12/13	0	36	Feline calicivirus (5)
	P289/290	5		
Poultry	JV12/13	0	66	Sapovirus-like sequences (7)
	P289/290	7		
	P289/290	32		

In Belgium, the apparent molecular presence of BoNV was 7.5% in diarrheic calf stools for 2007 (75) and 9.33% in diarrheic calves and young stock for 2008 (76) with GIII.2 being by far the most prevalent genotype in both studies. These prevalence data are in accordance with previously published studies from the United Kingdom, Hungary, Germany and South Korea. Other epidemiological data available from calf farms in industrialized countries indicate molecular detection levels ranging from 1.6 % up to 72%. Similarly to our results, a majority of GIII.2 was also observed in most studies and GIII.1 Jena virus detection was limited to Hungary, USA and South Korea. In 2007, no GIII.1 Jena virus was detected in Belgium. In order to exclude a technical artifact, a primer pair was especially designed upon the Jena virus sequence. Results in 2008 still confirmed a predominance of GIII.2 circulating in Belgian cattle. This observation supports the idea of the existence of predominating NV genotypes that dispose of advantages upon other genotypes similar to what was observed for GII.4 NVs in humans (115).

According to a 3 month survey in pigs in Belgium, 4.6% were found positive for PoNV in 2007 (74). Although molecular prevalence studies conducted in other industrialized countries ranged from 0.2 to 25%, our results are congruent with data from Hungarian, Canadian and Japanese pigs. PoNVs detected cluster into 3 genotypes within GII (131) and all three genotypes have been detected in Europe, America and Asia. Results from surveys seem to indicate that PoNVs exclusively infects finisher pigs (> 10 weeks old pigs) and for a great majority the detection of PoNV was not associated with any sign of enteritis. This is in agreement with data of experimental infections where PoNVs was responsible of mild diarrhoea in pigs (131).

Despite the fact that the epidemiological data available for NVs circulating in cattle and pigs seems to indicate that BoNVs and PoNVs are endemic in different parts of the globe, prevalence data diverges greatly between studies. The disparities observed could be mainly explained by i) the use of different detection methods, ii) the use of internal amplification controls for the detection of false negative results and iii) different sampling strategies. The clinical impact of BoNVs and PoNVs still remains unclear as few experimental data is available, for some survey studies no clinical data was available and for the majority of positive samples the enteric signs could be associated to other pathogens (75). This is true for all other animal NVs detected until now (68, 69, 135) with the exception of murine NVs that cause a fatal systemic disease in immunocompromised mice (51).
Studies have reported the detection of human-like NV sequences in porcine and bovine faecal samples and in a pork retail sample (73, 87) as well as antibodies against human NVs in pigs (36). Nevertheless, no evidence of transmission of NVs between domestic animals and human beings in natural conditions is available yet. For a better comprehension of NVs transmission and evolution, the continuous surveillance of these viruses in animals and humans will be needed.

Human noroviruses

All clinical or food samples found positive or suspected to be positive were sent by partner 3 (IPH) to partner 1(Ulg) for further investigation. These clinical samples were sent to IPH in relation with the platform of foodborne outbreaks where the samples were screened for noroviruses by RT-qPCR. Genotyping of human NVs from outbreak samples was realized based upon partial polymerase sequences amplified for NV detection and partial and/or complete capsid sequences obtained with previously published primer pairs (44, 48, 53, 129) detailed in table XI.

Table XI: Primer pairs used for the detection and genotyping of human noroviruses.

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	-			
GISKF	CTGCCCGAATTYGTAAATGA	+	330	Capsid region	Kojima et al., 2002
GISKR	CCAACCCARCCATTRTACA	-			
GIISKF	CNTGGGAGGGCGATCGCAA	+	344	Capsid region	Kojima et al., 2002
GIISKR	CCRCCNGCATRHCCRTTRTACAT	-			
FW1	GCGATCGCAATCTGGCTCCCAG	+	1284	Capsid region	Kamel et al., 2009
RT5	AGGTGYACATTATGACCAGTTC	-			

Of the 104 fecal samples analyzed by C1, 65 were found positive for norovirus and 60 could genotyped either in the polymerase region and/or in the capsid region. Due to the discrepancy between the different genotyping methods described for NV in the literature

(84, 138, 141, 142) and the continuous emergence of new NV sub-genotypes, we decided to refer to the standardized nomenclature drafted by Noronet available at www.rivm.nl. Results from NVs genotyped between December 2006 and July 2010 are summarized in table XII.

Table XII: Genotyping results in the partial polymerase (B) and the capsid (C) regions for NVs detected in human feces from December 2006 to July 2010.

Year of collection	Sample reference		Genotyping	
2006	C1	P3	Region B	Region C
	ISP 55		GII.4 2006b	
	ISP 57		GII.4 2006b	GII.4 2006b
	ISP 59		GII.4 2006b	
2007	ISP 472	1105-2007-0472	GII.4 2006a	GII.4 2006a
	ISP 473	1105-2007-0473	GII.4 2006a	GII.4 2006a
	ISP 474	1105-2007-0474	GII.4 2006a	GII.4 2006a
	ISP 475	1105-2007-0475	GII.4 2006a	GII.4 2006a
	ISP 477	1105-2007-0477	GII.4 2006a	GII.4 2006a
2008	ISP 333	1105-2008-2159	GII.2	
	ISP 335	1105-2008-2161	GII.2	GII.2
	ISP 336	1105-2008-2162	GII.2	GII.2
	ISP 356	1105-2008-2204	GII.4 2008	GII.4 2008
	ISP 358	1105-2008-2206	GII.4 2008	GII.4 2008
	ISP 360	1105-2008-2252	GI.4	GI.4
	ISP 363	1105-2008-2255	GI.4	GI.4
	ISP 374	1105-2008-2700	GII.4 2006b	GII.4 2007
	ISP 375	1105-2008-2838	GII.e	GII.4 2007
			GII.4 2006b	
	ISP 379	1105-2008-3007	GII.6 ou 7	GII.6
	ISP 383	1105-2008-3059	GII.4 2006b	
	ISP 384	1105-2008-3060	GII.4 2006b	GII.4 2006b
	ISP 388	1105-2008-3064	GII.4 2006b	
	ISP 393	1105-2008-3069	GII.e	GII.4 2007
2009	ISP 407	1105-2009-0143	GII.e	GII.4 2007
	ISP 450	1105-2009-0608	GII.4 2006b	GII.4 2006b
	ISP 498	1105-2009-02171	GII.e	GII.3
	ISP 499	1105-2009-02172	GII.e	GII.3
	ISP 501	1105-2009-02174	GII.e	GII.3
	ISP 502	1105-2009-02175	GII.e	GII.3
	ISP 503	1105-2009-02176	GII.e	GII.3
	ISP 584	1105-2009-3075	GII.4 2010	GII.4

2010	ISP 617	1105-2010-00160	GII.4 2010	
	ISP 619	1105-2010-00162	GII.4 2010	GII.4 2010
	ISP 620	1105-2010-00163	GII.g	GII.1
	ISP 621	1105-2010-00164	GII.g	GII.1
	ISP 622	1105-2010-00165	GII.4 2010	GII.4 2010
	ISP 624	1105-2010-00167	GI.7	
	ISP 626	1105-2010-00251	GII.4 2010	GII.4 2010
	ISP 630	1105-2010-00294	GII.4 2010	GII.4 2010
	ISP 632	1105-2010-00296	GII.4 2010	GII.4 2010
	ISP 633	1105-2010-00297	GII.4 2010	GII.4 2010
	ISP 634	1105-2010-00298	GII.4 2010	GII.4 2010
	ISP 637	1105-2010-00301	GII.4 2010	GII.4 2010
	ISP 640	1105-2010-00310		GII.4 2010
	ISP 642	1105-2010-00312	GII.4 2010	GII.4 2010
	ISP 644	1105-2010-00314	GII.4 2010	GII.4 2010
	ISP 647	1105-2010-00317	GII.4 2010	GII.4 2010
	ISP 648	1105-2010-00505	GII.g	
	ISP 649	1105-2010-00506	GII.4 2010	GII.4 2010
	ISP 652	1105-2010-00671	GII.4 2010	GII.4 2010
	ISP 653	1105-2010-00748		GII.13
	ISP 654	1105-2010-00966	GII.g	GII.1
	ISP 655	1105-2010-00967	GII.g	GII.1
	ISP 656	1105-2010-00968	GII.g	
	ISP 657	1105-2010-01010	GII.4 2010	GII.4 2010
	ISP 660	1105-2010-01093	GII.7	
	ISP 1143	1105-2010-01143	GII.2	GII.2
	ISP 666	1105-2010-01919	GII.g	GII.1
	ISP 667	1105-2010-01920		GII.1
	ISP 670	1105-2010-01936	GII.g	GII.1
	ISP 671	1105-2010-02020	GII.g	GII.1
	ISP 672	1105-2010-02021	GII.g	GII.1
	ISP 673	1105-2010-02022	GII.g	GII.1
	ISP 674	1105-2010-02023	GII.g	GII.1

All food samples sent by IPH failed for genotyping probably due to a lack of genetic material in the samples. This point was particularly limiting for the study of NV transmission routes as the implications of found in the outbreaks could not be confirmed by molecular analysis.

STUDY OF NOROVIRUS RECOMBINATION

Strains clustering differently based upon the region taken into account are considered to be recombinant. Efforts were put upon the amplification of regions in the NV genome at both sides of the ORF1/ORF2 junction in order to detect recombinant strains. Phylogenetic analyses were conducted in MEGA software and breakpoints were visualized by similarity plots.

Animal noroviruses

With the combined primer pair CBECu-F/AMG1-R, no amplicon was obtained for bovine samples BV164, 168 and 362 where discrepancy between polymerase and capsid clustering was observed. However, a 2,410-nucleotide (nt)-long genomic sequence was obtained from BV416 (GenBank accession number FJ946859) with CBECu-F/TVN-linker, which was a recombinant sequence genetically related to the Thirsk10 strain (94). Recently, similar chimeric GIII.1-GIII.2 NVs have been described in Norway (45) indicating these viruses might be widespread and predominate upon its parental GIII.1 Jena virus.

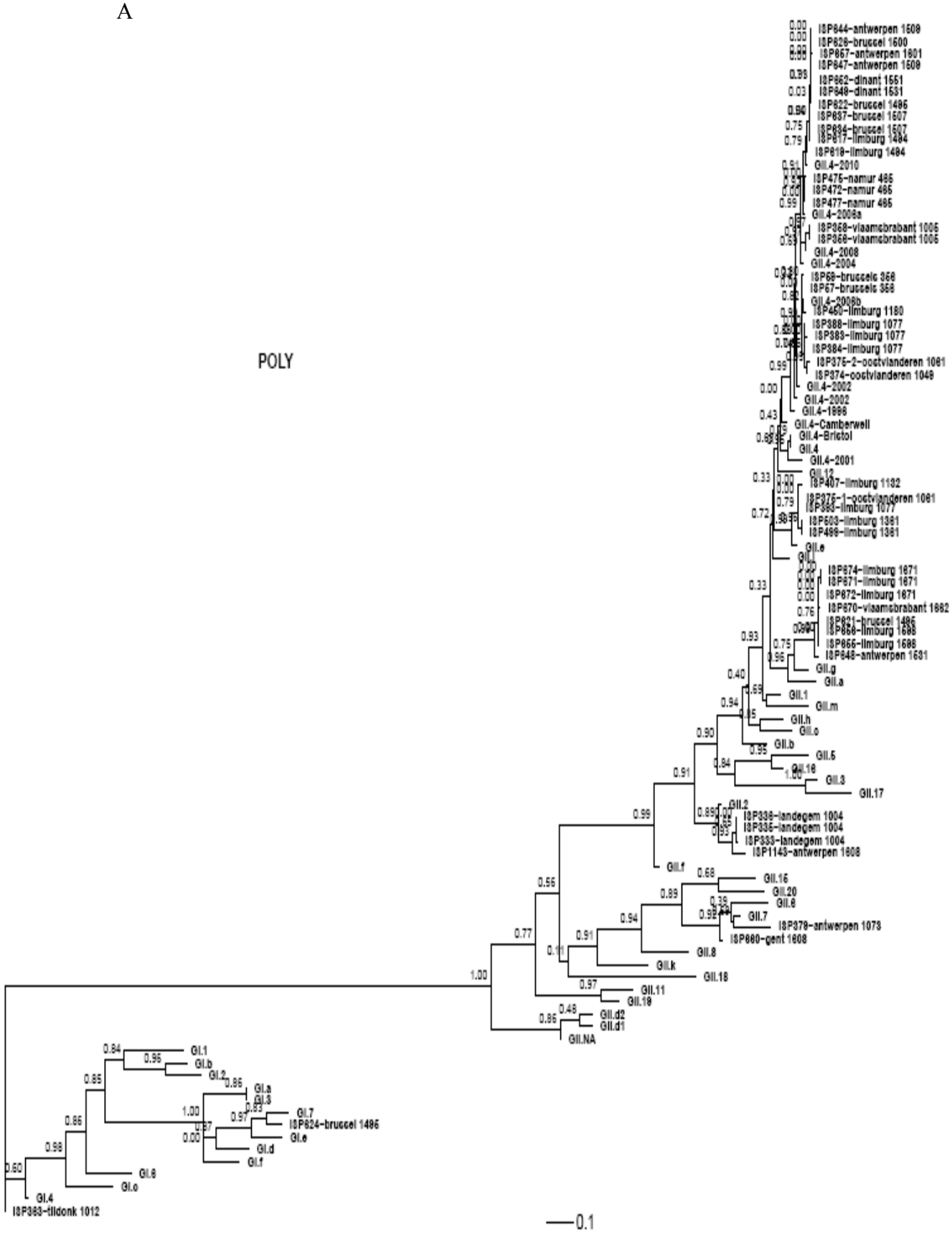
In our study, the 2 porcine norovirus strains clustered within GII.19 genotype and were detected in feces from two asymptomatic finisher pigs. Although ORF1/ORF2 recombinant PoNVs have been described before (131), no recombination event could be evidenced in the PoNVs detected in our study.

Human noroviruses

Incoherent clustering, as determined by the RIVM/Noronet genotyping tool was observed for multiple NV strains and these strains have been highlighted in grey in table XII. Phylogenetic trees were drawn and confirm different clustering depending on the region analysed (figure 6). Furthermore, they allowed the genotyping of NV strains that could not be assigned by the Noronet genotyping tool leading to the exclusion of strains that were believed to be recombinant (ISP 356-358: GII.4 2008). Results indicated that recombinant NVs were involved with at least 9 different outbreaks throughout 2006-2010 and exhibit different mosaics in their genome (GII.4 2006b/GII.4 2007; GII.e/GII.4 2007; GII.e/GII.3; GII.g/GII.1). For several of these natural recombinant NV strains, sequences covering the ORF1/ORF2 junction were amplified (ISP407 (GII.e/GII.4 2007), ISP 499-502 (GII.e/GII.3), ISP 620-673 (GII.g/GII.1) and recombination sites could be localised by Simplot analysis (figure 7). It was not possible to realise a similarity plot for the recombinant sequence ISP407 as all sequences available for the GII.4 2007 capsid in the Genbank database are associated with a GII.e polymerase. For the GII.g polymerase, sequences with either GII.12 or GII.13 capsids have been published (33, 41) allowing Simplot analysis for ISP620 and ISP673.

Sequences obtained in the human and bovine clinical samples show different NV strains that exhibit incoherent clustering for the partial sequences of the polymerase and the capsid region indicating that they might be recombinant. For the human NV strains, although the majority of the gastroenteritis outbreaks were involved with GII.4 NVs in 2007 and 2008, other GII NVs were detected from the end of 2008 to 2010 along GII.4 NVs. Among these NVs, a variety of new recombinants were detected in different samples from different outbreaks between 2008 and 2010. New « super » polymerase sequences (GII.e and GII.g) related to the previously described GII.b polymerase were detected in the same period. The exact significance of the emergence of these polymerases or their origin has yet to be elucidated but their involvement with different outbreaks might indicate that they have a selective advantage upon the capsid parental strains.

A


POLY
ISP844-antwerpen 1609
ISP826-brussel 1600
ISP867-antwerpen 1601
ISP847-antwerpen 1609
ISP862-dinant 1551
ISP848-dinant 1631
ISP822-brussel 1486
ISP837-brussel 1607
ISP834-brussel 1507
ISP817-limburg 1484
ISP818-limburg 1484
GII.4-2010
ISP475-namur 485
ISP472-namur 485
ISP477-namur 485
GII.4-2006a
ISP368-vlaamsbrabant 1005
ISP366-vlaamsbrabant 1005
GII.4-2008
GII.4-2004
ISP68-brussels 368
ISP67-brussels 368
GII.4-2006b
ISP460-limburg 1180
ISP388-limburg 1077
ISP383-limburg 1077
ISP384-limburg 1077
ISP375-2-oostvlanderen 1081
ISP374-oostvlanderen 1048
GII.4-2002
GII.4-2002
GII.4-1996
GII.4-Camberwell
GII.4-Bristol
GII.4
GII.4-2001
GII.12
ISP407-limburg 1132
ISP376-1-oostvlanderen 1081
ISP383-limburg 1077
ISP603-limburg 1381
ISP499-limburg 1381
GII.e
GII.i
ISP874-limburg 1871
ISP871-limburg 1871
ISP872-limburg 1871
ISP870-vlaamsbrabant 1882
ISP821-brussel 1486
ISP866-limburg 1686
ISP866-limburg 1686
ISP848-antwerpen 1631
GII.g
GII.a
GII.1
GII.m
GII.h
GII.o
GII.b
GII.5
GII.16
GII.3
GII.17
GII.2
ISP338-landegem 1004
ISP336-landegem 1004
ISP333-landegem 1004
ISP1143-antwerpen 1808
GII.f
GII.16
GII.20
GII.8
GII.7
ISP379-antwerpen 1073
ISP880-gent 1608
GII.8
GII.k
GII.18
GII.11
GII.19
GII.d2
GII.d1
GII.NA
GI.1
GI.b
GI.2
GI.a
GI.3
GI.7
ISP824-brussel 1486
GI.e
GI.d
GI.f
GI.8
GI.o
GI.4
ISP363-tildonk 1012
0.1

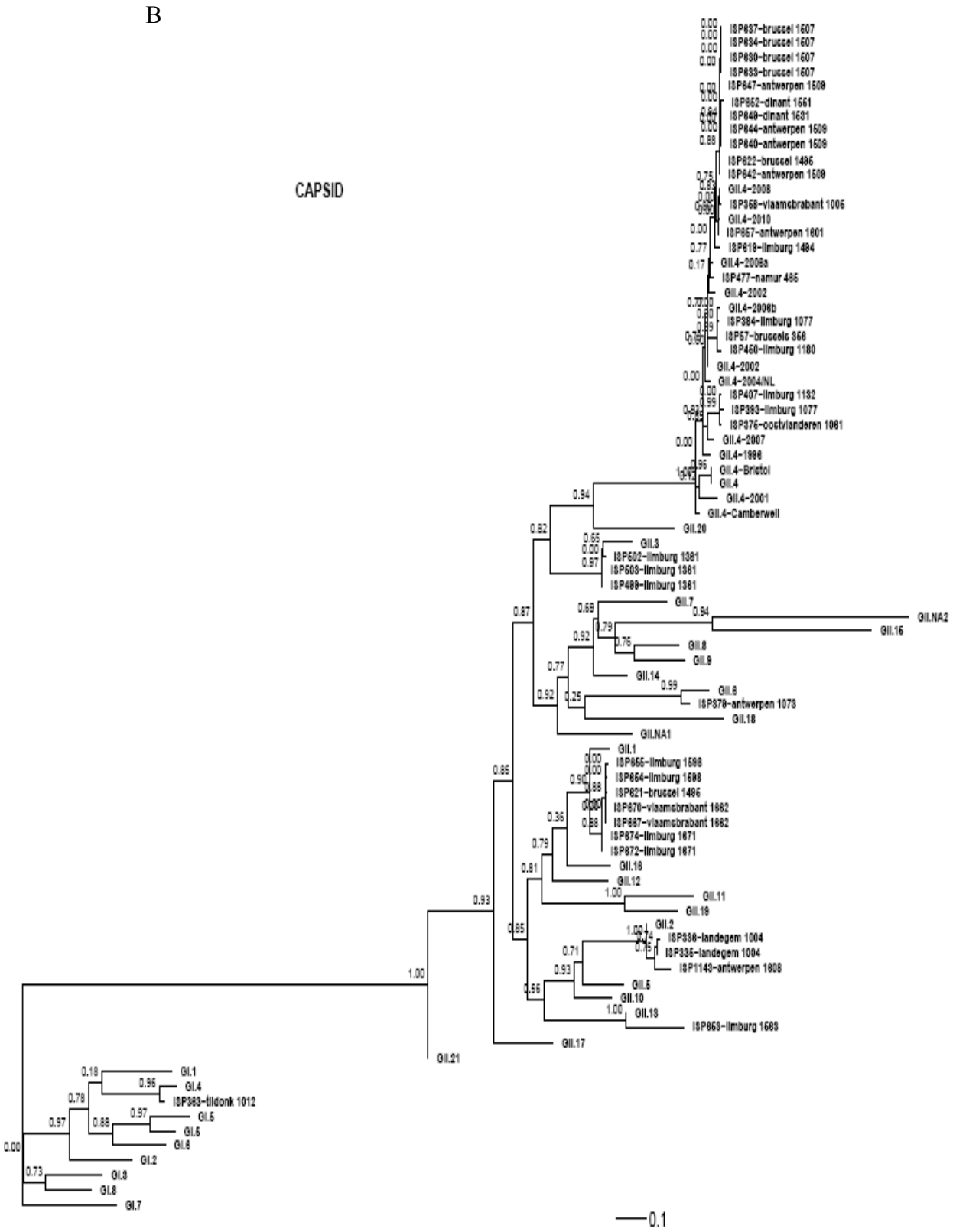


Fig 6: Phylogenetic analysis of the nucleotide sequences corresponding in the partial polymerase gene **(A)** and the partial capsid gene **(B)** with reference sequences proposed by the National Institute of Public Health and the Environment, The Netherlands, www.rivm.nl.

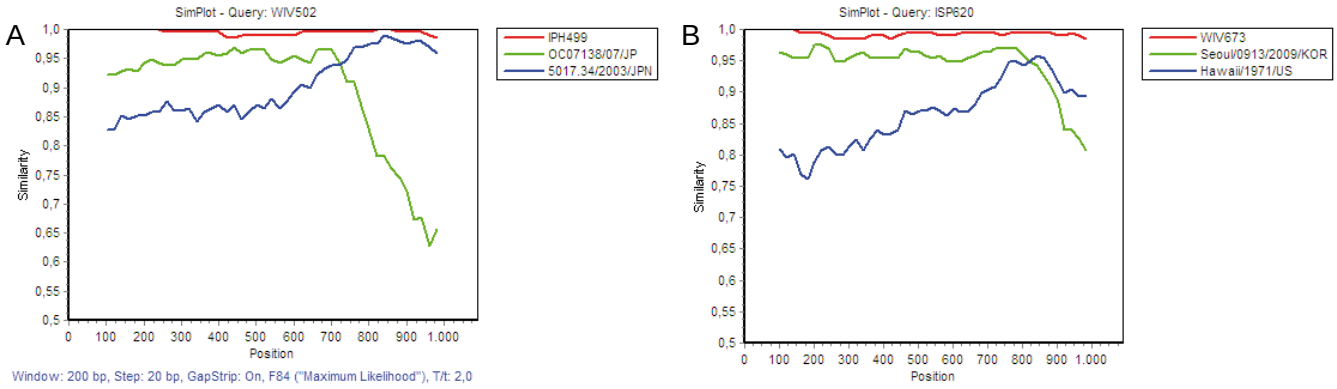


Fig 7: Similarity plot for ISP502 (and 499) and ISP 620 (and 673). A) The graph represents as a percentage the identity of the 2 putative parental strains, OC07138/07/JP (green line) and 5017.23/03/JP (blue line), with the recombinant strain ISP502. The window size was 200 bp with a step size of 20 bp. 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. The percentage identity of ISP499 (red line) to ISP502 is also plotted. B) The graph represents as a percentage the identity of the 2 putative parental strains, Seoul913/09/KOR (green line) and Hawaii/71/US (blue line), with the recombinant strain ISP620. The window size was 200 bp with a step size of 20 bp. 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. The percentage identity of ISP673 (red line) to ISP620 is also plotted.

In vitro study of recombination in murine Noroviruses (MNVs)

The discovery of a cultivable murine NV in 2003 has given the opportunity to study the biology of NV through this model. Up to date the description of recombinant NV strains limited to the phylogenetic analysis of different parts across the NV genome due to the lack of an efficient cell culture system for human NVs.

Here, two different MNV-1 strains have been chosen to study *in vitro* recombination, MNV-1 and WU20 (122), according to different important criteria. Isolates should: 1) be easy to grow in RAW cells, 2) form large lytic plaques on RAW cells monolayers enabling virus isolation, 3) have full-length published sequences, 4) be genetically closely related to each other allowing homologous recombination but 5) bear sufficient genetic markers for discrimination. Prior co-infections, genomic stability in cell culture was shown for both isolates after 4 successive passages and genetic markers distinguishing both isolates were identified. Based upon these markers, 3 real-time RT-PCR methods were designed to discriminate between both isolates in three different regions of the genome (N-term protein (5'), polymerase and ORF3 (3') in order to identify recombination events.

Raw cells were co-infected with both isolated at different MOI, different proportions, different environmental conditions. Progeny viruses from co-infection were either

analyzed directly or passed 4 times on cells before analysis. Virions were isolated by a plaque-picking method and characterized by the 3 genotyping qRT-PCR. Discrepancy in typing between these 3 regions will lead to further investigations for the characterisation of potential recombinant NVs. In order to confirm that recombination had occurred, the predicted recombination breakpoint of the potential recombinant MNV isolate (Rec MNV) and its parental viruses was sequenced. In order to investigate the effect of the recombination event on viral fitness, phenotypic characteristics of Rec MNV were investigated in cell culture. Single-step growth kinetics of the recombinant and parental strains were established from three independent series and the size of the plaques for each virus was determined.

Three discriminative real-time RT-PCRs were developed to discriminate between the parental MNV-1 viruses CW1 and WU20. Examples from discriminative screening are shown in figure 8.

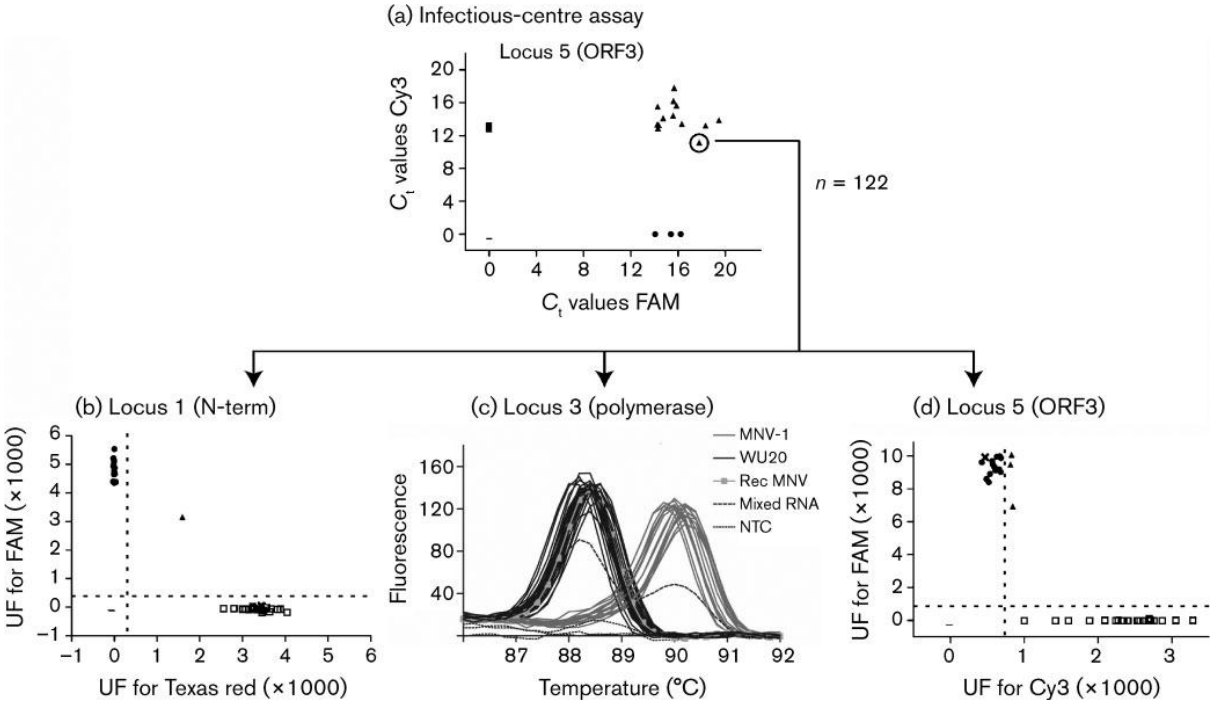


Fig 8: Detection of a recombinant MNV from a co-infected cell determined by an infectious-centre assay. (a) Twenty infectious centres were selected randomly from RAW cell monolayers previously co-infected by MNV-1 (●) and WU20 (■) at a total m.o.i. of 100 (50 each). ▲Mixed RNA; NTC, non-template control (–). Both parental genomes could be detected in 13 of 20 infectious centres. Of 122 progeny viruses from one co-infection scenario that were genotyped by PCR in three genomic regions [N-term (b), polymerase (c) and ORF3 (d)], only one virus (Rec MNV; x) showed discordant genotyping in the three regions. Ct, Threshold cycle; UF, units of fluorescence.

Results of the screening of the progeny viruses after different conditions of co-infection are represented in figure 9.

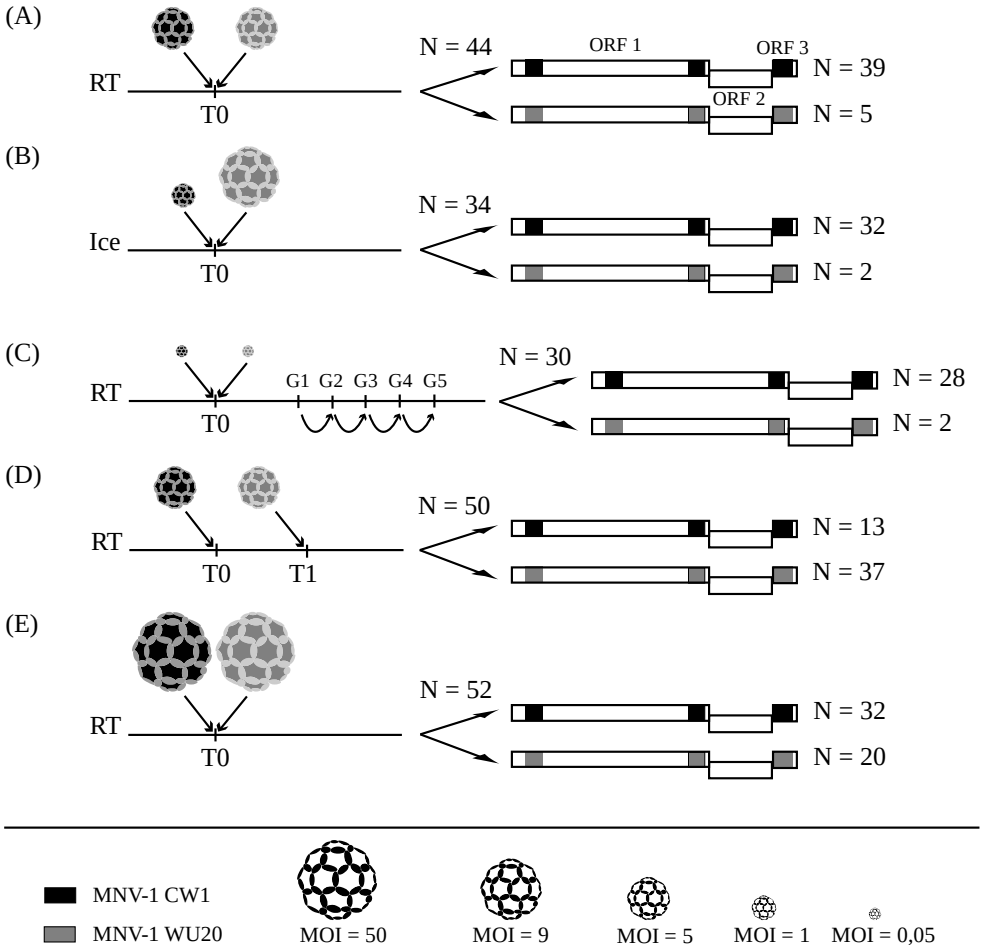


Fig 9: Schematic representation of different co-infections conditions between the MNV-1 CW1 and WU20 parental isolates and the results of the screening of the progeny viruses based upon discriminative real time PCR for 3 loci of the MNV-1 genome.

All progeny viruses were screened in 3 regions of the genome by discriminative real-time RT-PCR (N-terminal protein, polymerase and ORF3). A total of 210 progeny viruses have been characterized after co-infection. No recombinant viruses have been detected even when high MOI levels corresponding to high selective pressure was applied. In order to determine if RAW cells could be simultaneously infected by two viruses an infectious centre assay (ICA) was realized (Figure 8a). Results of discrimination on the progeny viruses from the infectious centres (plaques) formed by one cell indicated RAW cells could be co-infected by CW1 and WU20. We further analyzed 122 progeny viruses emerging from one co-infected cell in the 3 regions of the genome. One recombinant virus was detected based upon the discriminative RT-PCR results showing amplification for WU20 in the N-term and polymerase region although amplification for

CW1 was observed in the ORF3. These results indicated that recombination could have occurred between the polymerase region and ORF3.

ORF1/ORF2 junction has been identified in the norovirus genus as being a recombination hotspot (12). Therefore, we amplified a 1578 pb long fragment covering this junction from the potential recombinant progeny virus together with the parental viruses (CW1 and WU20) and cloned it into a plasmid vector before sequencing. Alignment of these sequences confirmed recombination and located the recombination breakpoint at the ORF1/ORF2 junction where a 123 pb sequence of complete homology could be observed (Figure 10). This observation does not allow us analyse more precisely the breakpoint.

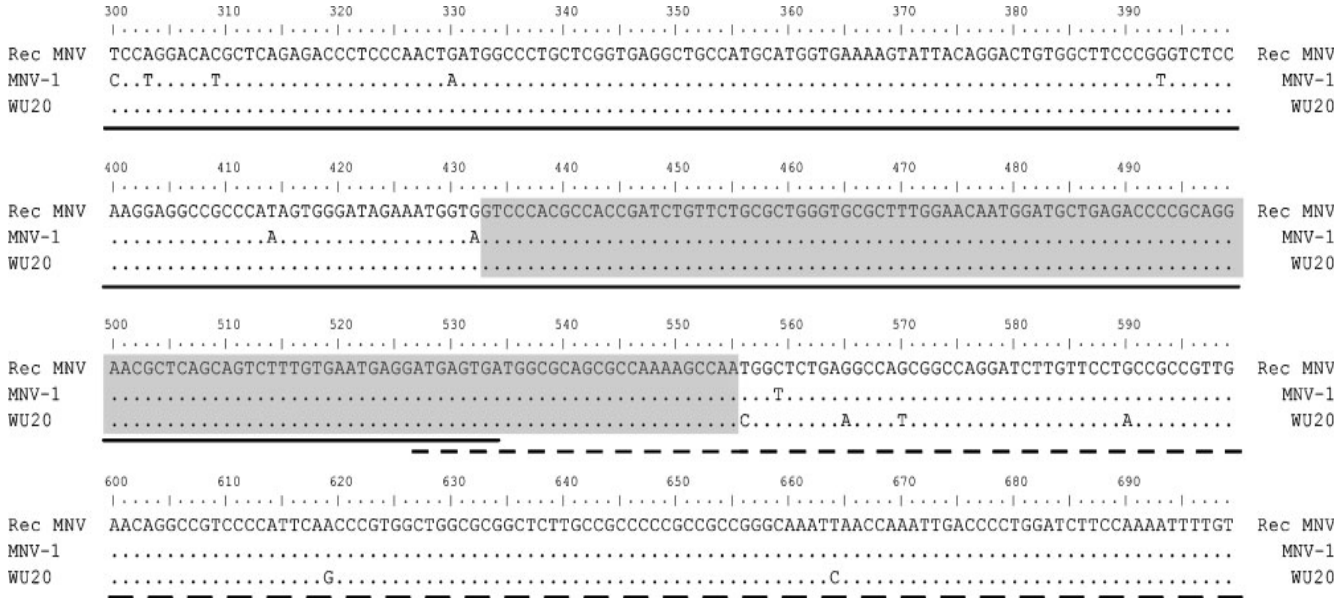


Fig 10: Sequence alignment of a 1530 bp fragment covering the ORF1–ORF2 junction of MNV recombinant (Rec MNV) and parental (MNV-1 and WU20) viruses. Nucleotide identities with the upper sequence are represented by dots. The boxed grey area represents the region (123 bp) that potentially served as breaking point in the RNA recombination event. Sequences corresponding to the 39 end of ORF1 and the 59 end of ORF2 are indicated by a continuous and a dashed line, respectively.

Whilst the three viruses showed similar growth curves when total and extracellular virus titres were analysed, differences were observed for intracellular virus production (Fig. 11a). For the parental viruses, intracellular virions constituted the majority of their total virus titres up to 18 h post-infection (p.i.) before extracellular titres exceeded the intracellular titres, probably due to lysis of the infected cells. In contrast, intracellular Rec MNV titres were maintained at a high level up to 24 h p.i. (Fig. 11a). Phenotypic characterization of Rec MNV was completed by plaque-size assays. In order to

determine the relevance of the differences in plaque size, a non-parametric statistical method that would take into account the variation in plaque size for each virus was chosen and data were analysed with the Kolmogorov– Smirnov statistic. Results obtained from 64 randomly selected plaques for each virus indicated that Rec MNV produced significantly smaller plaques than the parental isolates, with P-values ,0.05 (Fig. 11b, c). Taken together, these results indicate that, although similar total virus titres were obtained for all three viruses, Rec MNV seemed to be sequestered longer inside the cell before release. This longer cell association may reduce the spread of Rec MNV to neighbouring cells, thus explaining the smaller plaques. Thus, plaque-size analysis together with intracellular growth-curve kinetics of Rec MNV, in comparison with those of the parental viruses, indicated a reduction in fitness in vitro, probably due to less efficient virus egress.

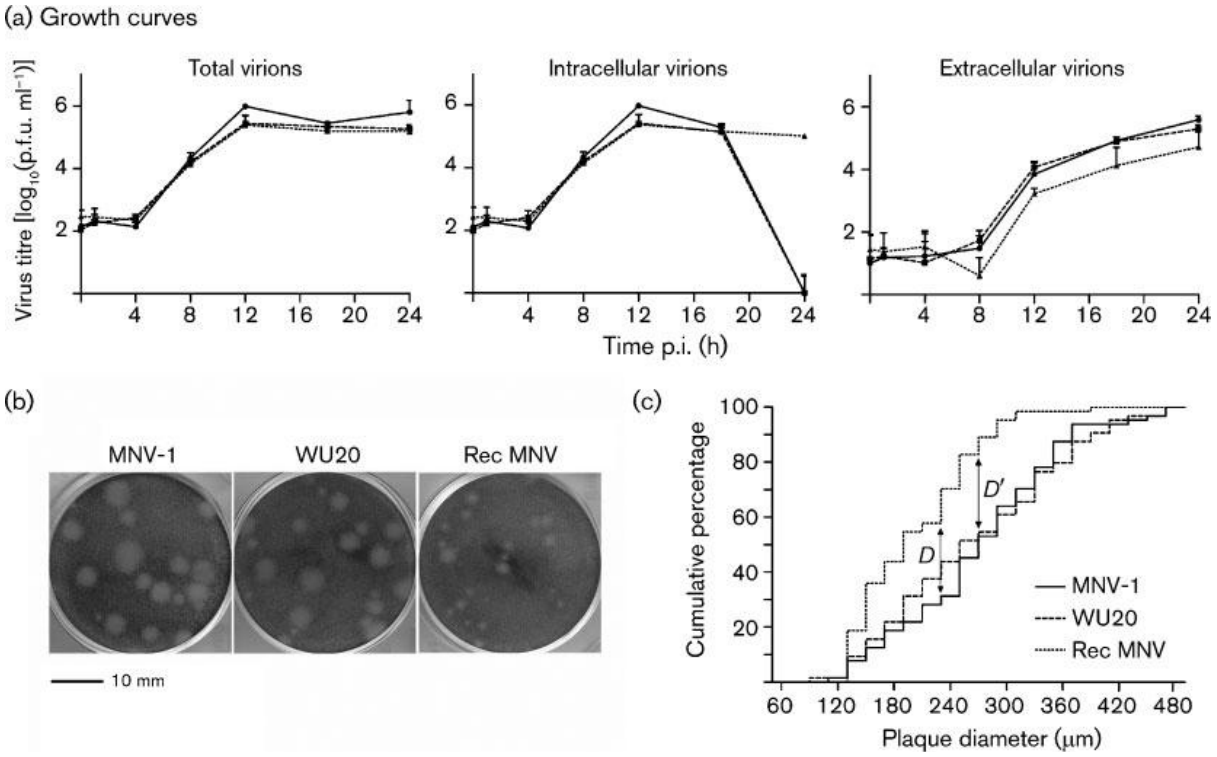


Fig. 11. In vitro growth properties of Rec MNV. (a) Single-step growth kinetics of MNV-1 (●), WU20 (■) and Rec MNV (▲ Data for total, intracellular and extracellular MNV virions were obtained after infection of RAW 264.7 cells at an m.o.i. of 5. (b) RAW cell monolayers were infected with dilutions of MNV-1, WU20and Rec MNV (passages 5, 4 and 3, respectively) and processed by plaque assay. (c) Plaque sizes of Rec MNV were compared with the parental ones by Kolmogorov–Smirnov statistic. Two-tailed P-values were used to determine the level of significance between the compared populations (86). The D calculated for Rec MNV/MNV-1 gave a P-value ,0.0001, and the D9 for Rec MNV/WU20 gave a P-value ,0.0011. For MNV-1/WU20, the Dmax value gave a P-value of 0.9497.

In conclusion, a recombinant virus was generated by coinoculation of RAW cells with two distinguishable MNV isolates in the absence of selection markers. The MNV model appears to be suitable for the study of NV recombination in cell culture in the absence of available culture systems for human NVs. Additional in vitro and in vivo studies would enable further insights into NV genetic-diversifying mechanisms such as recombination.

Work package 3: Risk profiling

DETECTION OF NOROVIRUS IN FOOD

In conclusion, an unexpected high presence of NV was observed by real-time RT-PCR in particular in fruit (raspberries, strawberries and cherry tomatoes) despite the good bacteriological quality. However, it should clearly be noted that these positive real-time PCR results do not provide direct evidence for the presence of infectious NV particles on the contaminated food products, since (q)PCR can only detect genomic material and thus cannot distinguish infectious and non-infectious NV particles. Therefore, results obtained during achievement of the fourth objective of the NORISK project show the difficulty of expressing positive (real-time) PCR results towards terms of public health threat if no associated diseases or outbreaks are reported. Although these low NV levels might indicate virus contamination at some point during the fresh produce chain, care should be taken to translate these results as a significant risk to the public health. Nevertheless, a possible risk for food borne transmission of NV from these food products cannot be excluded either.

For a better comprehension of NV transmission routes, sequences of the detected NVs in food and clinical samples were determined and submitted for further analysis. Genotyping of NVs in food matrices came out to be a real challenge and consisted into a bottleneck as the amount of genetic material on food was insufficient for PCR amplification and sequencing. This obstacle was not overcome during our project and NV sequences were only obtained from clinical samples in humans and animals.

ROLE OF THE ANIMALS IN NOROVIRUS TRANSMISSION

Until today all sequences obtained from human clinical samples clustered with human NV sequences and most NVs detected in animals, although related to HuNVs, clustered into genotypes or genogroups proper to each species. Even if the majority of the data available today tends to exclude interspecies transmission, several findings are in favour of its occurrence. The latter will be presented according to the four steps involved with the emergence of host-switching viruses: exposure, infection, spread and adaptation (99).

Exposure. NVs have been detected in humans all over the world, Bo- and PoNVs were evidenced in different geographical locations across the globe whereas canine NV (CaNV) detections were limited until now to the south of Europe. NVs were detected in domestic and wild animals in close contact with humans either kept for subsistence (cattle, pigs, sheep), ornamental (lion cub), research (mice) or companionship (dogs) purposes. Persons at particular risk would be not only animal owners (e. g. dog owners and farmers) but also people who are in contact due to professional activities (e. g. veterinarians, butchers, scientists, zookeepers and slaughters). Although no animal NVs have been detected in humans, Widdowson and collaborators (132) suggested IgG reactivity to BoNVs to be more common in veterinarians than in the control population. Another study showed that work tending to livestock and the presence of a dog in the close surrounding of the house were associated with increased odds for a seroresponse to NVs (101). These results indicate that some populations might be at risk and be more susceptible to be infected by animal NVs. NVs are oro-faecally transmitted and very resistant in environmental conditions. Consequently, apart from direct contact, indirect transmission can occur through the ingestion of contaminated recreational or drinking waters (infecting both animals and humans) or the consumption of contaminated bivalve shellfish. Indeed shellfish are capable of accumulating both human and animal NVs (23, 139) even though specific binding of BoNVs to oyster tissus was suspected to be less efficient. Whether NVs can be transmitted by produce of animal origin remains unclear. Human-like NV sequences were detected in retail pork meat but the source of contamination remained unclear and could originate from food handling (73).

Infection and spread. No animal NVs have been detected in human clinical samples until date and it is unclear whether animal NVs can infect humans. Serological studies showed higher antibody titres against recombinant capsid proteins of BoNVs in veterinarians than in the control group suggesting infection by BoNVs in this risk population (132). Although contradictive results were obtained for cross-reactivities between HuNV and BoNV epitopes (8, 40, 93, 132), this phenomena cannot be ruled out. Indeed, the higher prevalence of IgA against NV found in persons with dogs in their near environment or with regular interactions with lifestock could be explained by the existence of cross-reactive epitopes between human and animal NVs. Provided that results were interpreted with care, these data would suggest the possibility of animal NVs to infect humans. Carbohydrate antigens of the histo-blood group family, known to be shared among various mammal species, have been shown to act as receptor for different caliciviruses including NVs (109, 113, 120).This characteristic could enhance interspecies, including zoonotic, NV transmissions. Conclusions from virus-host interaction studies evaluate chances of BoNVs to infect humans to be thin seeing that the alphaGal epitope involved with BoNV VLP binding is not expressed in tissues of

human or porcine origin (140)(Mauroy in preparation). Similarly, porcine GII.11 epitopes failed to fix on human tissues and saliva whereas fixation was successful on stomacal and intestinal tissues of porcine and bovine origin (36) (thesis Zakhour 2009).
On the other way round, HuNV-like sequences have been detected in porcine and bovine fecal samples (73, 87, 125) but if HuNVs replicated in the animals remains unclear. Under experimental conditions, human GII NVs successfully infected gnotobiotic young pigs and calves and infections were accompanied by mild clinical signs limited to the intestinal tract (16, 116, 117). These results are in favour of the possibility of HuNV to sporadically infect these animals. To comfort this hypothesis, healthy cows' colostrums were shown to contain antibodies able to react to HuNV VLPs (85). Serological evidence of GIV NV infections were found in cats and dogs in Italy, again cross-reactions with other NV strains could not be ruled out (29).

Adaptation. Even if sporadic cross-species transmissions are common, a number of specific changes are required for the virus to infect, replicate and transmit efficiently in its novel host species. The probability of a virus to switch host species will depend upon different factors including i) virus potential, ii) host susceptibility or iii) the chance of occurrence. In this part we will develop more specifically the ability of viruses to successfully adapt to a new host. From an evolutionary point of view, viral variability is thought to enhance the probability of viruses to adapt to a new host (99). In this context, RNA viruses, due to a high number of nucleotide substitutions, could be more inclined to transgress host barriers. These high mutation rates are imputable to the error prone replication of RNA viruses with a lack of proofreading activity of the polymerase, the rapid virus replication with short virus generation times and the creation of large virus populations. NVs do not constitute an exception and high evolutionary rates of 10^{-2} to 10^{-3} nucleotide substitution/site/year were found (114, 128). On top of small-scale mutations, recombination and reassortment events have been largely implicated in the generation of viral diversity. Genetic reassortment or recombination could enable the creation of new combinations of genetic materials, generating more dramatic genomic changes than point mutations. Phylogenetic analyses of NV genomes have shown that recombination is likely to occur both in human and animal NVs (12, 69, 94, 122). Furthermore, experimental evidence of NV recombination was provided by an in vitro co-inoculation study (70). This study showed that a NV recombination event could yield a recombinant virus exhibiting biological properties that differ from the parental ones. Similarly, reassortment seems to play a crucial role in host switching for influenza A viruses. Thus, the fact that NVs can undergo genomic reshuffle through recombination could enhance their adaptation to a new host after cross-species transmissions.
In conclusion, NV zoonotic transmissions were observed neither during this project nor in the literature even if they cannot be excluded. Data available today suggest that

genetic recombination could lead the emergence of NVs capable of crossing host barriers or exhibiting modified virulence. Figure 12 shows the potential co-infection scenarios between human and porcine NVs that could lead to the generation of recombinant NVs with altered/enhanced biological properties.

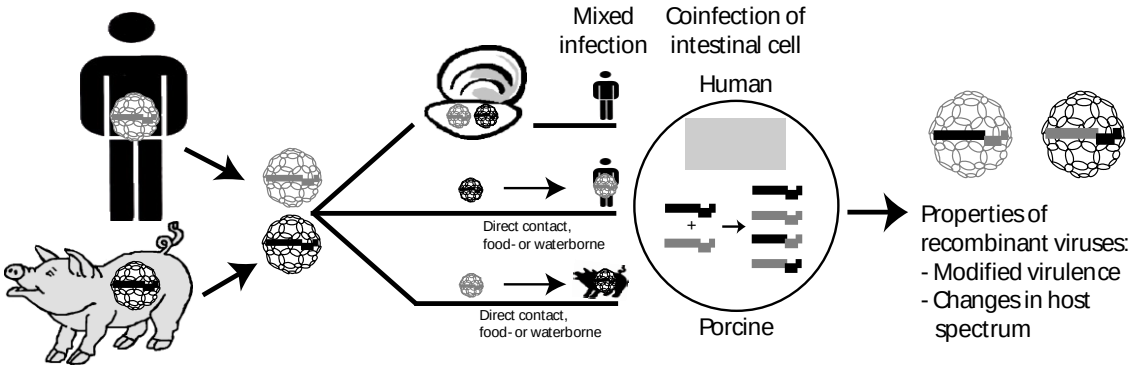


Fig 12: Schematic representation of how genetic recombination between human and porcine NVs could influence the biological properties of NVs namely the zoonotic potential or virulence.

Work package 4: Development of a network

In **Belgium**, it has been observed that in 20 to 50% of the reported outbreaks the causative agent remains unknown. NV is known to be an important cause of foodborne outbreaks and could be responsible for a part of these unknown cases. Besides the fact that only since 2006 an extraction and detection system for the detection of this virus was available for routine analyses in different kinds of foodstuffs, the actual number of NV infections is still underestimated because of a low reporting rate since the infection is normally self-limiting and complications are not known (65). Since 2007, human samples (feces) are also investigated for the presence of NV. However, in many outbreaks no patient samples were analysed for NV because those analyses were not reimbursed by social security in Belgium. A convention between the Flemish Community and the National Reference Laboratory for Foodborne Outbreaks in Brussels allowed human sample analysis in outbreaks which are suspected to be due to NV. However, the number of cases remains underestimated because not every ill person will visit a physician nor will all physicians request a stool sample, while not every patient will provide a sample if this is requested. In cases of outbreaks analysis of food samples is not always possible because no leftovers of the food are present. In some cases food of the same production date is analysed but this batch does not always has the same way of conservation or manipulation as the consumed food responsible for the outbreak.

In **2004**, two general foodborne outbreaks due to NV were registered in Belgium. In total 33 persons became ill after a restaurant visit, but the implicated foodstuff could not be identified.

In **2005** one NV outbreak was reported in a holiday park where 65 persons became ill after consuming a buffet meal in a restaurant. Epidemiological investigation assumed that the implicated foodstuff was the pizza served in the restaurant, however, NV was only detected in human samples.

In the beginning of 2006 a NV extraction and detection protocol was introduced in the laboratory for foodborne outbreaks. The extraction method procedure described by Baert et al. (4) was used.

In **2006**, 3 NV outbreaks were reported to the National Reference Laboratory for Foodborne Outbreaks (NRL-FBO). Two of these outbreaks occurred in a care centre for disabled persons. During the first episode, 12 persons became ill and NV (Genotype II) was found in one of the witness meals as causative agent. Four months later, 50 persons became ill in the same institute and a mixture of NV GI and GII was found in one of the witness meals analyzed. The origin of the disease and the transmission route were not proven but epidemiologically evidence demonstrated that food was probably the vehicle for transmission to the different members in this closed community.

A third outbreak took place in a hospital where 17 out of 400 people became ill. NV GII was detected in the soup and also in 5 out of 6 faecal samples. An infected person distributing the soup could have been at the origin of this infection. Generally, NV was found in 2.5% of the reported foodborne outbreaks in 2006. In all 3 NV outbreaks, the agent was detected in the food samples analyzed.

Eleven foodborne outbreaks out of the 75 foodborne outbreaks reported in Belgium during **2007**, were suspected for NV as causative agent. In eight of these outbreaks a food handler was involved and in the 3 remaining outbreaks bacterial pathogens were absent. Using the laboratory and epidemiological information 10 out of the 11 suspected foodborne outbreaks were confirmed as foodborne NV outbreaks, in the remaining outbreak food and clinical samples were negative for the presence of NV.

In total 392 persons were affected. Symptoms started in most cases between 12 and 24 hours after food consumption and in general vomiting, diarrhoea and slight fever was reported. Hospitalization was not necessary. The majority of outbreaks occurred at work (30%), the second most important settings were at camp (20%) and in nursing homes (20%) whereas one outbreak took place in a restaurant (10%), a recreation place (10%) and at home (10%).

In 8 outbreaks the food handler was suspected to be the source of the contamination. Stool samples were not always taken or, if so, these were not always tested for NV presence but tested negative for bacterial pathogens.

Epidemiological information suspected food handlers for the transmission of NV in several outbreaks, as the implicated food items were mostly manipulated and served afterwards by the kitchen personnel. Analysis of the individual ingredients reveal no NV contamination (no primary contaminated food) only the served composited meal tested positive for NV.

In two cases a known history of gastroenteritis was reported for the food handler. The first case considered a member of the staff of a restaurant, who suffered from gastroenteritis during the week before the outbreak occurred. Sandwiches prepared by the kitchen personnel, including this particular food handler, tested positive for the presence of NV. In a second case, a sick child assisted in preparing sandwiches at camp.

In one outbreak in a recreation park, NV was detected both in leftovers of the served food (soup, chicken and rice) and in the human fecal samples. An infected person serving the meal for the children was probably responsible for the contamination of the food. Once the children returned home, 34 persons more became sick having the same symptoms because of satellite outbreaks in the families.

In the other foodborne outbreaks mashed potatoes, meat stew and a composite meal were found positive for NV. In these cases no stool samples were available for detection of NV presence. In 40% of the outbreaks sandwiches were the vehicle of the NV outbreak. There was one suspected waterborne outbreak at a camping place in summer. Epidemiological information indicated tap water as the most suspected source of the outbreak. However due to the lack of an appropriate concentration and extraction method for NV detection in water, negative results were obtained.

In **2008**, the follow up of food-borne outbreaks was continued. In total 104 outbreaks were reported to the National Reference Laboratory and 999 people became ill.

In 11 of these outbreaks NV was detected as causative agent, with in total 439 ill people. Five of those outbreaks occurred in elderly homes where person to person contact was responsible for transmission of NV.

In the other 6 outbreaks food was the suspected source of the outbreak and samples were analyzed. In one outbreak, 200 out of 3000 exposed people became ill (diarrheoa, vomiting and slight fever) after eating sandwiches at a new year's event, where families of factory workers were invited to celebrate the beginning of the year. Fecal samples of different patients were found bacteriological negative, but tested positive for NV GII. Leftovers of the sandwich- spreads were also analyzed, but tested negative for both the presence of bacteria or NV. Contamination of the sandwiches with NV by a contaminated food-handler might be at the origin of the outbreak, however, no epidemiological evidence was available. Alternatively, transmission from person to person could have played an important role in spreading NV in this outbreak. A second outbreak occurred at a working place where 15 out of 70 exposed persons became ill

after eating sandwiches. Different fillings were tested and three samples were found positive (meatball, cheese and chicken curry) for the presence of NV. In this case the food handler who prepared the sandwiches was proven to be the source of infection, as a fecal sample of this person tested positive for NV. This food handler was not allowed to enter the kitchen area for one month, until a new fecal sample tested negative.
A third outbreak took place in September where 80 people participating at 3 different independent barbecues became ill. The food for these events was provided by the same butcher. There were no remarks on the hygiene practices in the butcher's shop. Several leftovers were tested for NV but all were found negative. In contrast, fecal samples from ill persons tested positive for NV presence. Interestingly, the fecal sample of the butcher's wife was also found positive. Indeed, in the butcher's family there was a history of gastroenteritis during the week before the outbreaks at the different barbecues took place. In this case the food handler was again very important in the transmission of the virus. Another outbreak occurred on a school trip where 47 children became ill. Although food was first thought to be at the origin of the outbreak, epidemiological information showed that in this case a vomiting child in the toilet, which was only cleaned with water instead of using a detergent, was at the origin of the outbreak.
Another NV outbreak was reported in a children's garden where 15 young children became ill. An institutional catering prepared dinner for this children's garden and also for other elderly homes in the same village. In the latter, no infections were observed, so the food was probably not involved and thus not analyzed. This means that in this outbreak the most important route of transmission was from person to person.
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 were negative for NV. One of the guests in the restaurant was suffering from gastroenteritis, but there was no evidence that this person was responsible for the transmission of NV to the other people present in the restaurant.
Based on epidemiological evidence, a last NV outbreak was reported on a cruise ship in Antwerp. No human or food samples were taken. In this case, only recommendations for cleaning and disinfection were provided to the cruise ships personnel.

A total of about 100 outbreaks have been reported to the NRL-VTI in **2009**, 8 of these reported outbreaks were confirmed for NV with a total of 95 ill persons. In none of these cases NV could be detected in both food and clinical samples. In 4 cases all food samples were found negative although NV could be detected in the patients' feces. In one case the coproculture of one foodhandler was shown to be positive for NV. In two cases no food matrices were analyzed and NV was detected in the feces samples. In these cases food was not thought to be the source of infection. Finally, in two other cases NV was detected in food samples but not in the clinical samples. In one case no feces have been sent for analysis and in the other case feces were found negative for

NV. For this last case other enteric viruses could be the cause of the outbreak. Like we already noted in the previous years, foodhandlers with gastroenteritis continue to constitute a risk to the transmission of the infection through manipulation and serving of the food. NV contaminated food sources were twice meals that were composed by various components (meat, vegetables and potatoes).

From preliminary data in **2010,** 61 outbreaks have been reported to the NRL FBO. In 18 outbreaks (29%), NV was detected as causative agent. This caused 789 ill persons and 56 hospitalizations. One person with an underlying pathology died during a NV outbreak on a cruise ship. In two outbreaks with a co-infection, both NV and a bacterial germ were at the origin of a foodborne outbreak. In this case, another 360 ill persons and 31 hospitalizations were noted. In these cases it is not possible to give an exact number of ill people due to NV. Between 17/07/2010 and 30/07/2010, 4 summer camps suffered from severe gastro-enteritis. A total number 196 persons became ill over a period of some days. At a camp in Dilsen-Stokkem, two subsequent outbreaks were reported within only 10 days. During the first outbreak at July 18th , 49 children of the 172 exposed persons became ill and 8 others were hospitalized. A second outbreak at the same location took place on July 27th, where 45 of the 183 exposed persons became ill. Here, 17 persons were hospitalized. A hamburger was found to be positive for NV, probably by manipulation of a infected person. In the same period, another 15 ill people were reported at a youth camp of 72 participants in Affligem. All ill persons slept in the same room and used common sanitary facilities. The water tested negative for NV. In a 4th outbreak in Kampenhout, 2 peaks of ill persons were reported in which 71 of the 133 exposed children became ill during the camp. Another 16 persons became ill during the following five days after the camp was ended. In this outbreak, 30 hospitalizations were required. For all outbreaks mentioned above, human and/or samples from the environment were found to be positive for the presence of NV. In particular, the samples from the commonly used places such as swabs taken from the sinks and tap in the sanitary room and toilet seats. Thanks to the results of environmental swabs, the origin of transmission and infection could be located and an effective cleaning and disinfection was possible. After disinfection, the environmental swabs resulted negative for the presence of NV and a subsequent youth camp could be organized.

Based on the experience acquired and on all the knowledge collected during foodborne outbreaks it was possible to write down a scenario specific for gastro-enteritis. The investigation and control of food-borne outbreaks is a multi-disciplinary task requiring information of different areas of clinical medicine, epidemiology, food microbiology, food safety and food control, risk communication and management. In Belgium, different authorities are dealing with food-borne outbreaks and as a consequence the information is dispersed and difficult to collect for reporting purposes. Investigations by the Federal Agency for the Safety of the Food Chain (FASFC) are mainly focused on food

related matters and the Communities (Flemish, French and German Community and Brussels) deal with person related matters like illness. Ideally, both authorities are involved in investigations of reported outbreaks. To have a better communication and exchange of information, the food microbiology laboratory of the Institute of Public health was designated in 2005 to be the National Reference Laboratory for food-borne outbreaks (NRL-FBO). It collects, centralizes the information, analyzes all suspected food samples and does the reporting and the follow-up of the FBO in Belgium. NV transmission is complex and this scenario for gastroenteritis is important for a better classification of the outbreaks based on the transmission routes.
A complete scenario for gastroenteritis outbreaks focused on NV outbreaks is added in annex 3

Table XIII: Overview of reported NV outbreaks from 2004 to 2010 at the NRL FBO

	2004	2005	2006	2007	2008	2009	2010
Total reported outbreaks (>2 ill)	57	105	116	75	104	96	61
Total number ill	531	673	1032	846	841	857	1159
Total Norovirus outbreaks	3,5%	0,9%	3,4%	13,3%	10,6%	8,3%	29%
Number of ill due to Norovirus	6,2%	9,6%	14,9%	46,3%	52,2%	11%	68%

Observations made in the outbreaks reported during the past 4 years have lead to several recommendations in terms of disinfection management in order to limit NV transmissions.
Because NV is mainly transmitted from person-to-person, environmental samples of surfaces within commonly used places (toilets, sinks, doorknobs, kitchen surfaces, …) often lead to the origin of transmission of NV. A good diagnostic allows reacting straightforward in case of NV outbreaks and helps to prevent secondary infections. This demonstrates the need for preventive measures at public places and in locations where food is manipulated (37, 54, 80)(Codex Committee on food hygiene). NV is highly persistent and viral RNA can be present for 4 weeks at a surface (62). Moreover, NV is very infectious and only few viral particles (~10) are likely to be sufficient to cause an infection (121)(www.cdc.gov). Therefore, it is of high importance to disinfect the environment after cleaning the surface with soap and warm water. Due to the high resistance of the virus to high temperatures and a low pH, numerous alternative methods (irradiation, exposure to high pressure) and disinfectants were tested to inactivate NV. Because NV is not cultivable in the laboratory, these inactivation studies are performed on closely related viruses such as the Feline Calicivirus (FCV), Murine NV

(MNV) or on Hepatitis A virus (HAV) (2, 3, 15). These studies demonstrated that a solution of ≥1000 ppm (parts per million) free chlorine result in a decrease of 3 $\log_{10}$ in viral titer for HAV and FCV (Codex Committee on Food Hygiene). Moreover, only disinfection using 1000 ppm free chlorine could avoid transmission from contaminated surfaces (37). For the preparation of a 1000 ppm solution, 1 chlorine tablet (1.5g free chlorine) should be resolved in 1.5 liters of water, or 16 ml bleach (NaOCl, 20°) per liter water should be used. Because of stability reasons, the use of tablets is recommended. Depending on the type of surface to be disinfected, different concentrations of chlorine are used (see table). If a low concentration of chlorine must be used for reasons of the type of material, a repetition of disinfection with a lower concentration is recommended. Alternatively, the surface can be treated with steam for materials which do not support any chlorine treatment. Other practical measures can be taken such as the use of disposable towels in public toilets, a good hand hygiene and regular cleaning of the surroundings to decrease the spread of NV (www.cdc.gov; (37)). In the case of close contact with other persons or when food is manipulated with bare hands, a good hand hygiene is recommended. Therefore, cleaning hands with regular liquid hand soap is recommended as it inactivates NV in a more efficient way as compared to alcohol-based disinfectants (9, 63). Because of its resistance to temperatures up to 65°C, tissues (clothes, sheets) contaminated with NV should be washed once at 90°C or twice at 70°C (www.rivm.nl).

Table XIV: Overview of the use of chlorine for disinfection of NV *

Chlorine (ppm**)	Application
200 ppm	Stainless steel Food/mouth contact items Toys
1000 ppm	non-porous surfaces tile floors counter-tops Sinks Toilets
5000 ppm	Porous surfaces Wooden floors

*from Centre for Disease Control – World Health Organization

**: parts per million

In outbreaks, the identification of infected persons is not only of interest to avoid further transmission from person-to person or via the surroundings, but also are at the origin of food contamination. A study based on international literature and the Belgian data demonstrate that in 42.5% of the NV outbreaks between 2000 and 2007 a contaminated food handler was at the origin of the outbreak (6). Therefore, it is recommended that a food handler or other persons with clinical symptoms of a (viral) gastro-enteritis are

prohibited from the work floor or the group event to avoid spread of the infection (Moe *et al.,* 2009; Codex Committee for Food Hygiene). An important reduction in the number of NV outbreaks was observed when nurses or visitors from elderly homes were forbidden to enter in case of gastro-enteritis symptoms (37). Even after recovery from the disease, shedding of NV is possible during 2 or 3 weeks (54, 80, 106). Asymptomatic infections have also regularly been observed (54). This means that healthy asymptomatic carriers are able to spread the virus in the surroundings (56).

3. POLICY SUPPORT

The presence of Mieke Uyttendaele, Georges Daube, Lieve Herman, Katelijne Dierick and Etienne Thiry in the scientific committee of the Belgian Food Safety Agency (AFSCA-FAVV) allowed the input of the expertise acquired during this project for the answering of questions asked to the agency.

The advice of the HGR-CSS "Viruses in food" nr 8386: This publication reports the epidemiological situation at national level and gives recommendations for future scientific research. This report includes the main characteristics of foodborne viruses, the answers to questions that could be asked by competent authorities and optimal management of outbreaks.

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 Agency for the Safety of the Food Chain 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.

On request of the Health inspection, a publication on the detection and prevention of Norovirus was submitted (Vlaams Infectieziektenbulletin). In parallel, a short communication will be presented in the LabInfo journal of the Federal Agency for Safety of the Food Chain.

4. DISSEMINATION AND VALORISATION

General conclusions of the NORISK project will be presented during a meeting that will be held on Tuesday 5th of April 2011 at the Scientific Institute of Public Health before the meeting for the launching of the newly created Belgian Society for Food Microbiology

Active membership/participation in different working groups:

- COST929 – Environet: A European Network for Environmental and Food Virology (Ugent, ULg)
- CEN WG6 TAG4: A European standardisation working group developing a two-part (quantitative and qualitative) standard method for virus detection in foodstuffs, including shellfish, which has the potential to be incorporated into EU legislation as a reference method (IPH, ULg)

Poster presentation and abstract (in book of abstracts)

Typage moléculaire des norovirus bovins isolés en Belgique : caractérisation génomique d'un isolat de type Newbury-2.
Scipioni A., Mauroy A., Ziant D., Czaplicki G., Lomba M., Thiry E.
- IXèmes Journées Francophones de Virologie, April 2007, Paris, France.

Novel two stage real-time RT-PCR assay to detect and quantify human and bovine noroviruses.
Scipioni A., Mauroy A., Ziant D., Vinjé J., Thiry E.
- 26th Annual Meeting of the American Society for Virology, July 2007, Corvallis, Oregon, USA.
Norovirus bovins isolés en Belgique en 2007 et investigation de leur potentiel zoonotique par l'étude des interactions virus-cellules.
Mauroy A, Scipioni A., Mathijs E., Thys C., Thiry E.
- Xèmes Journées Francophones de Virologie, March 2008, Paris, France.

Détection de calicivirus félin dans des matières fécales félines et canines.
Mathijs E., Zicola A., Mauroy A., Scipioni A., Thys C., Thiry E.
- Xèmes Journées Francophones de Virologie, March 2008, Paris, France.

Norovirus and sapovirus in pigs in Belgium.
Mauroy A., Scipioni A., Mathijs E., Miry C., Ziant D., Thys C., Thiry E.

- COST929-Symposium : Current developments in food and enviromental virology, 9-11 October 2008, Pisa, Italy.

Multiplex real-time RT-PCR for simultaneous detection of GI/GII noroviruses and murine norovirus 1.
A. Stals, H. Werbrouck, L. Baert, N. Botteldoorn, E.Wollants, L. Herman, M.Uyttendaele and E. Van Coillie.
- 14th PhD symposium on Applied Biological Sciences. Belgium (Ghent), 14/09/2008
- Benelux qPCR symposium, poster and abstract. Belgium (Ghent), 06/10/2008.
- 1st COST929 Symposium "Current Developments in Food and Environmental Virology". Italy (Pisa), 9-11/10/2008
- Rapid Methods Europe 2009. The Netherlands (Noordwijk), 26-28/01/2009.
- KVCV - Trends in food analysis VI. Belgium (Ghent), 19/05/2009.
- Research seminar. Belgium (Ghent), 24/02/2009.

Evaluation of a norovirus detection methodology for soft red fruits
A. Stals, L. Baert, E. Van Coillie and M. Uyttendaele.
- 14th conference on food microbiology. Belgium (Liège), 18-19/09/2009.
- 2009 IAFP European Symposium. Germany (Berlin), 7-9/10/2009

Evaluation of a norovirus detection methodology for ready-to-eat foods
A. Stals, L. Baert, A. De Keuckelaere, E. Van Coillie and M. Uyttendaele.
- 15th conference on food microbiology. Belgium (Liège), 16-17/09/2010.
- Exchange 2010. Belgium (Ghent), 28/09/2010
- 4th international conference on Caliciviruses. Chili (Santa Cruz), 16-19/10/2010.
- 2nd COST 929 symposium. Turkey (Istanbul), 7-9/10/2010.

Screening of fruit products for norovirus and the difficulty of interpreting positive PCR results
A. Stals, L. Baert, V. Jasson, E. Van Coillie and M. Uyttendaele.
- Food Micro 2010. Denmark (Copenhagen), 30/08/2010 – 03/09/2010.
- 15th conference on food microbiology. Belgium (Liège), 16-17/09/2010.
- Exchange 2010. Belgium (Ghent), 28/09/2010
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Murine NoV: a surrogate for NoV detection in bivalve shellfish?
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Mauroy A., Scipioni A., Mathijs E., Thys C. and Thiry E.
- 8th International Congress of Veterinary Virology, 23-26 august 2009, Budapest, Hungary

Oral presentation

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Scipioni A.
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Multiplex real-time RT-PCR for simultaneous detection of GI/GII noroviruses and murine norovirus 1.
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Detection of contemporaneous human and bovine noroviruses in Belgium.
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Evaluation of a norovirus detection methodology for ready-to-eat foods.
A. Stals, L. Baert, A. De Keuckelaere, E. Van Coillie and M. Uyttendaele.
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