AF/01

NORISK - Results

Transmission routes of Noroviruses, emerging human pathogens present in the food chain

DURATION OF THE PROJECT
01/01/2007 – 31/01/2011

BUDGET
569.911 €

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.

CONTEXT

Noroviruses (NV) 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.

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.

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.

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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 104 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..

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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.

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