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TRANSMISSION ROUTES OF NOROVIRUSES, EMERGING HUMAN PATHOGENS IN FOOD

"NORISK"

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FINAL REPORT
SUMMARY

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

SD/AF/01A

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Janvier 2009

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Elisabeth Mathijs, Etienne Thiry, Georges Daube, Ambroos Stals, Lieve Herman, Mieke Uyttendaele, Nadine Botteldoorn, Katelijne Dierick. ***Transmission routes of noroviruses, emerging human pathogens in food "NORISK"***. Final Report Phase 1 Summary. Brussels : Belgian Science Policy 2009 – 6 p. (Research Programme Science for a Sustainable Development)

CONTEXT

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

OBJECTIVES

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

WORKPLAN

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

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

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

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

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

RESULTS-CONCLUSIONS

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

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

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

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

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

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

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

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

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

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

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

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

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

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

RECOMMENDATIONS

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