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Fourth Nordic Workshop on Food and Waterborne Viruses

Anna Charlotte Schultz and Birgit Nørrung

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Nordic co-operation

Nordic cooperation is one of the world's most extensive forms of regional collaboration, involving Denmark, Finland, Iceland, Norway, Sweden, and three autonomous areas: the Faroe Islands, Greenland, and Åland.

Nordic cooperation has firm traditions in politics, the economy, and culture. It plays an important rol in European and international collaboration, and aims at creating a strong Nordic community in a strong Europe.

Nordic cooperation seeks to safeguard Nordic and regional interests and principles in the global community. Common Nordic values help the region solidify its position as one of the world's most innovative and competitive.

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Preface

Food and water borne viral infections caused by norovirus (NoV) and hepatitis A virus (HAV) are known causes of human diseases. In particular noroviruses are recognised as the most common microorganisms associated with outbreaks of acute viral gastroenteritis in the Nordic countries and transmission through food and water is well documented. Several foodborne outbreaks of acute viral gastroenteritis in the Nordic countries have been linked to imported fresh produce and the risk of viral foodborne infections may increase with the increased international trade of fresh produce.

The Nordic countries – Denmark, Finland, Iceland, Norway and Sweden – often share the wishes for the same food products and with the trade agreements and co-operation in many areas, the food area is to a large extent subject to the same legal requirements in the Food acts and in the political, democratic decision-making process in these countries. Official opinions in one country within an area do often raise questions in the other countries. In recent years, the initiative for co-operation between the Nordic countries has increased considerably as to the exchange of information on outbreaks, prevalence and research on food and waterborne viruses. With Denmark, Finland, Sweden and Estonia being members of the European Union, and Iceland and Norway being associated through the European Economic Agreement (the EEA agreement), the subject of food and waterborne viruses was dealt with in a workshop financed by the Nordic Council of Ministers.

The main objective of this workshop was to exchange information of updated Nordic and International research activities regarding the diagnostic experience of enteric viruses in food and water and to renew and maintain contacts between Nordic experts in this field. In addition, it was the aim to discuss possibilities for future project collaborations between the Nordic countries in order to obtain a joint unit containing different expertises. Such collaborations could enhance the methodology development and bring us further towards standardised detection methods to be used in routine testing laboratories and surveillance programs in the Nordic countries.

During the workshop international leading experts presented their latest results within the area of food and waterborne viruses. The current situation regarding outbreaks, epidemiology and research in the Nordic countries was presented. The exchange of information gave rise to discussions and recommendations for future work. This report is proceedings from the workshop and includes extended abstracts and presenta-

tions[1] of the speeches. References are only included in some chapters, where only main references are given.

The workshop participants consisted of the following persons (see annex G for more detailed personnel data):

Denmark

Anna Charlotte Schultz, Jeffrey Hoorfar, Birgit Nørrung, Peter Saadbye and Dorte Lau Baggesen, Institute of Food and Veterinary Research (DFVF), *Blenda Böttiger*, Staten Serum Institut (SSI), *Linda Bagge* Danish Environmental Protection Agency (MST) and *Anders Dalsgaard*, The Royal Veterinary Agricultural University (KVL).

Estonia

Natalia Kerbo, Health Protection Inspectorate.

Finland

Leena Maunula University of Helsinki *and Leila Rantala* National Veterinary and Food Research Institute (EELA).

Iceland

Anna Pála Vigdisdóttir, Environment & Food Agency of Iceland (UST), *Sigrun Gudnadóttir*, University Hospital of Iceland.

Norway

Mette Myrmel and Ann Kristin Øye, The Norwegian School of Veterinary Science. Inger Lill Anthonisen, Sykehuset i Vestfold HF, and Karin Nygård, Nasjonalt folkehelseinstitutt.

Sweden

Kjell-Olof Hedlund, Marika Hjertqvist, Maria Lysén and *Yvonne Andersson*, Swedish Institute for Infectious Disease Control. *Flemming Lund,* National Food Administration.

[1] The full report including presentations can be found at: http://www.norden.org/pub/sk/showpub.asp?pubnr=2006:584

International expert speakers:

Spain
Albert Bosch, University of Barcelona.

United Kingdom
Sam Bark, Centre for Environment, Fisheries and Agricultural Science (CEFAS).

France
Soizick Le Guyader, IFREMER.

Nederland
Wim van der Poel, Wageningen University Research.

USA
Jan Vinjé, Center for Disease Control and Prevention, Atlanta.

Anna Charlotte Schultz, DFVF, has directed the project. Birgit Nørrung and Jeffrey Hoorfar, DFVF, and Jan Vinjé, CDC, have chaired the workshop. Workshop participants from the individual Nordic countries and international invited experts have made the abstracts and presentations for the workshop.

The Nordic Committee of Senior Officials for Food Issues under the Nordic Council of Ministers has sponsored this report.

Summary

On June the 1st–2nd 2004 the Danish Institute of Food and Veterinary Research (DFVF) hosted a Nordic Workshop on food and waterborne viruses. The workshop took place at Tune Landbrugsskole. Like previous Nordic workshops within this field, held in Sweden, Finland, and Norway in the years 1996, 1997 and 2000 respectively, this present workshop was financed by the Nordic Committee of Senior Officials for Food Issues under the Nordic Council of Ministers. The aim of the workshop was to renew and maintain contacts between Nordic experts and to exchange and discuss information on the present situation regarding epidemiology and methods for the detection of human enteric viruses in food and water.

At the workshop 30 participants from 11 countries were present. Next to the Nordic countries and Estonia, International colleagues from Spain, France, The Netherlands and USA contributed with their expertise.

Presentations illustrating the current Nordic situation on food and waterborne viral outbreaks, epidemiology and diagnostics were given by participants from the Nordic countries. The International experts covered the main research areas in focus by presenting updates in relation to clinical means of human enteric viral infections, the methodology for the detection of viruses in food and water matrices and the potential for zoonotic transmission. In addition, a promising tool for microbiological source tracking of fecal pollution in water and shellfish was presented. The presentations contributed as a basis in the information exchange and discussions.

The main conclusions from the workshop were:

- Sensitive and quantitative detection methods as well as genotyping methods need to be further developed and validated.
- Epidemiological studies including case control studies must be prioritised.
- The role of animal and environmental factors in transmitting enteric viruses needs to be further studied.
- Information accumulated in the Nordic countries should be used in a joint formal risk assessment.
- The risk of transmission of food borne enteric viruses through the lack of general hygiene measures needs to be communicated to food handlers and consumers.
- It is of great importance to maintain/strengthen the Nordic Network created through previous years workshops on viruses in food and water. This networking facilitates new and more concrete internordic network and research project collaborations.

- Evaluation of detection methods for viruses in foods is an ongoing activity in several Nordic countries. Collaboration and a joint Nordic funding would strengthen these activities. In addition this could improve our influence in the International working groups connected to the development of method standards (CEN/TC275 WG6/TAG4) and shellfish monitoring systems (Reference Laboratory network for microbiological monitoring of shellfish), where problems are discussed and decisions are taken.
- The Nordic shellfish industry is among the largest in Europe and it is presently in a huge growth. It is therefore of great importance that we participate in the research activities of virus detection in foods in order to participate in the decision making processes regarding systems for virus monitoring including selection of methods for detection of viruses in foods.

Sammendrag

Den 1. og 2. juni 2004 var Danmarks Fødevareforskning, DFVF, vært ved en nordisk workshop omhandlende fødevare og vandbårne virus. Workshoppen fandt sted på Tune Landbrugsskole. Tidligere nordiske workshops/seminarer indenfor samme område blev afholdt i årene 1996, 1997 og 2000 af hhv. Sverige, Finland og Norge. I lighed med disse blev også denne »Fourth Nordic Workshop on Food and Waterborne viruses« finansieret af EK-Livs under Nordisk Ministerråd. Formålet med workshoppen var at forny eller fortsætte kontakter mellem nordiske eksperter på området samt at opdatere, udveksle og diskutere informationer om den nuværende situation vedrørende epidemiologi og metoder til påvisning af virus i vand og fødevare.

På workshoppen var der 30 deltagere fra 11 lande. Udover deltagere fra de nordiske lande og Estland deltog også foredragsholdere fra Spanien, Frankrig, USA, Holland og England.

De Nordiske deltagere præsenterede status om forholdende vedrørende fødevare og vandbårne virus udbrud samt epidemiologi og diagnostik i de enkelte lande. Præsentationer fra de internationale eksperter gav en bred opdatering på forskningsområder i fokus omkring klinisk betydning og udbredelse af de relevante virusinfektioner, nyeste metoder til detektion af disse i vand og fødevarer, samt potentialet for zoonotisk transmission. Endelig blev der præsenteret et lovende værktøj der muliggør mikrobiologisk kildesporing af fækal forurening i vand og skaldyr. Præsentationerne udgjorde grundlaget for informationsudveksling og diskussioner.

Hovedkonklusionerne fra workshoppen var følgende:

- Der er brug for forskning indenfor udvikling og validering af diagnostiske redskaber til påvisning af virus i vand og fødevarer. Kvantitative påvisnings metoder bør udvikles og valideres.
- Epidemiologiske studier herunder case control studier bør prioriteres.
- Betydningen af miljømæssige faktorer og zoonotisk transmission af enteriske virus bør studeres nærmere.
- Informationer opsamlet i de nordiske lande bør samles i en fælles formel risikovurdering.
- Det er vigtigt at kommunikere om risikoen for overførsel af virus via fødevarer ved dårlig hygiejne til fødevarehåndtører og forbrugere.
- Det er vigtigt at fastholde og styrke det nordiske netværk dannet gennem de foregående års workshops på virus i fødevare og vand.

Traditionen faciliterer nye og mere konkrete internordiske netværk og projektsamarbejder.

- Evaluering af virus detektions metoder er allerede en del af de nordiske landes arbejdsområder. Samarbejde og fælles finansiel støtte fra Nordisk side vil styrke indsatsen. Dette vil også styrke indflydelsen i de internationale fora f.eks. Inden-for metode standardisering (CEN/TC275 WG6/TAG4) og skaldyrsmonitorerings systemer (Reference Laboratorie netværket for mikrobiologisk monitorering af skaldyr), hvor problemstillinger bliver diskuteret og beslutninger bliver truffet.
- Den nordiske skaldyrsindustri er en af de største i Europa og er i disse år endvidere i kraftig vækst. Derfor er det af afgørende vigtighed at vi er med til at træffe beslutninger omkring monitoreringssystemer, herunder de metoder der i fremtiden bliver valgt til anvendelse ved påvisning af virus.

1. Introduction

Several groups of enteric viruses may infect persons after ingestion and then be spread via stool and vomit. Among these, norovirus, NoV, (formerly Norwalk-like viruses) and hepatitis A virus, HAV, are currently recognised as the most important foodborne pathogens with regard to the number of outbreaks and people affected in the Western world. These viruses are, as far as currently known, all of human origin.

Outbreaks with NoV normally give rise to a great number of cases compared to other foodborne pathogens due to the high susceptibility of NoV in the population and a high rate of secondary infections. In 2002, NoV was estimated to be the leading foodborne agent in USA. CDC has characterized NoV as an “emerging pathogen”, as it accounts for more than 65% of non-bacterial gastroenteritis outbreaks. Worldwide, approximately 7% of reported HAV cases have been associated with shellfish consumption, and in Italy HAV accounts for 43% of all registered cases. At present, NoV is the most common pathogen causing foodborne outbreaks in Sweden. During 2000–2003, there was an annual average of 18 (range 12–28) foodborne outbreaks reported with approximately 800 cases (range 331–1212) each year; in total 74 outbreaks with 3183 cases. During the past years imported NoV contaminated frozen raspberries and oysters have caused several outbreaks in Finland, Sweden and Denmark. In Finland, Sweden and Norway NoV contaminated drinking water has led to several outbreaks. In addition other types of foods, such as ready-to-eat foods, cold prepared menu ingredients at restaurants or from caterings have increasingly been incriminated as vehicles of virus transmission. In these cases the origin of contamination is often due to insufficient hygiene preparation by infected food handlers or kitchen staff. In general most foodborne outbreaks are identified through epidemiological data coupled with the clinical identification of the virus suspected from the history of symptoms of the infected persons. In some cases kitchen staff or food handlers are being examined if they are suspected to be the course of virus transmission.

Methods for the detection of enteric viruses in food and water

The direct detection of virus in food and water are for many reasons a great challenge. First of all Norovirus can not be grown in cell culture and culturing of hepatitis A is difficult and time consuming. Several mo-

lecular methods have been developed for the purpose, but they often include limitations in their ability to isolate and detect the often few particles that may be present in the food item. During the past years viral detection methods have been described mostly to be used for shellfish, but also to a lesser extent other food items. In some cases these methods has shown to be applicable to detect viruses in connection with outbreaks. All these methods rely on molecular detection in the form of PCR-techniques or RT-PCR (reverse transcriptase - polymerase chain reaction), where the sequences of the genome (DNA or RNA) are duplicated to a detectable level. It is however doubtful if these methods are capable of the detection of virus in levels below 100 particles per gram. In addition, several factors as controls for extraction efficiencies of the viral genome, avoidance of cross contamination during the PCR process, optimal RT-PCR conditions etc. must all be taken into consideration before a reliable and sensitive result is achieved. Analyses for the detection of enteric viruses in foods are therefore cumbersome and expensive, which presently makes them unsuitable for use in routine control laboratories.

Faecal indicators

In recent years a number of studies have been performed to compare Norovirus occurrence with indicator organisms such as the traditionally used E. coli and different bacteriophages. In several studies of shellfish it was shown that the presence of male specific F^+ RNA phages (FRNA) was more often correlated with the presence of viruses than E. coli. However, the absence of such FRNA phages was no guarantee for the absence of viruses in shellfish product. For monitoring of the safety of shellfish regarding the risk of viral infections we therefore seem to be left with the detection of viruses themselves. Nevertheless interest has been shown on the possibility to use the distribution of FRNA sero- or genotypes as a tool for microbiological source tracking. An issue that has gained increased attention lately among researchers.

2. Enteric viruses

This chapter contains international research areas presently in focus on enteric viruses and their importance in foodborne transmission. It includes abstracts of the presentations given at the workshop by the invited experts within the specific areas. The related presentations are shown in appendix C.

2.1 Food- and waterborne viruses – a general overview

Blenda Böttiger
Statens Serum Institute (SSI), Copenhagen, Denmark.

Food- and waterborne viral infections are increasingly recognised as causes of illness in humans. The most commonly implicated pathogens in Scandinavia at present are caliciviruses, especially noroviruses, and to a lesser degree hepatitis A virus, but other viruses such as enteroviruses, rotaviruses, adenoviruses and astroviruses can also be spread by water and food. Clinical and epidemiological aspects of these viral infections will be presented.

During the last three years an EU-financed project "Food-borne viruses in Europe" has existed aiming at optimizing and harmonising the detection of viral food- and waterborne outbreaks in the 9–11 participating countries. By facilitating sharing of laboratory and epidemiological data widespread, international outbreaks have been detected. Novel variants of noroviruses have also been detected - initially as the cause of water- and foodborne outbreaks but later also as the cause of other outbreaks in the countries involved. Another important observation has been that contaminated food and water can contain multiple lineages of norovirus, and infecting people with a range of different virus types. Hence, sets the stage for generating new virus types by recombination of viral genomes.

2.2 Enteric viruses in water

Albert Bosch
University of Barcelona, Barcelona, Spain.

Pathogenic viruses are routinely introduced into environmental waters through the discharge of treated and untreated sewage, since current water treatment practices are unable to provide virus-free wastewater effluents. It is estimated that the number of cases of gastrointestinal illness annually reported worldwide accounts for billions, while enterically transmitted hepatitis outbreaks, i.e. hepatitis A and E, have been reported to be associated to water and shellfish.

The basic steps of the virological analysis of water are sampling, concentration, decontamination/removal of inhibitors, and specific virus detection. Concentration is a particularly critical step since the viruses may be present in such low numbers that concentration of the water samples is indispensable to reduce the volume to be assayed for viruses to a few milliliters or even microliters.

A good concentration method should fulfill several criteria: it should be technically simple, fast, provide high virus recoveries, be adequate for a wide range of enteric viruses, provide a small volume of concentrate, and be inexpensive. In relatively nonpolluted waters, the virus levels are likely to be so low that optimally hundreds, or even thousands, of liters should be sampled to increase the probability of virus detection. Nevertheless, all available methodologies have important limitations. The efficiency of a virus concentration method is widely depending on the quality of the sampled water. Usually, all available procedures have been basically evaluated in spiked samples, and the recovering efficiencies recorded with experimentally contaminated water dramatically decrease when the method is applied in actual field trials. Additionally, none of the existing concentration procedures has been tested with all the groups of medically important viral pathogens; normally, a few specific enteric viruses have been employed to conduct the evaluation trials.

The advent of molecular techniques for virus detection, and particularly reverse transcriptase-polymerase chain reaction (RT-PCR), provided exquisite tools for the detection of fastidious health-significant viruses in the water environment, which were previously unrecognizable because they replicate poorly or not at all in cell cultures. One limitation of molecular techniques is that they fail to discern between infectious and non-infectious particles, which may be of critical relevance in environmental virology. One possibility to solve this problem, at least for some specific virus strains, may be the use of cell lines susceptible to support the propagation of a wide variety of enteric viruses, enabling the amplification

of virus sequences in cell culture prior to detection by PCR, accomplishing the dual purpose of increasing the number of copies of target nucleic acid and of incorporating an infectivity assay as well.

A recent improvement comes from the emergence of combined rapid thermocycling and fluorescence monitoring of amplified product, collectively referred as “rapid-cycling real-time PCR”, together with nucleic acid sequence-based amplification or NASBA techniques, both of which now applicable in several commercially available systems. These procedures enable not only qualitative determination but also, and particularly, quantitative diagnostic assays. Although the generic determination of pathogens is the essence of diagnostic practices, the possibility to quantitatively detect virus agents represents a seminal refinement in routine monitoring virology.

However, in the meantime and due to technical difficulties, tests for most of these viruses remain restricted to laboratories with sophisticated facilities and well-trained personnel. On the other hand, it is impracticable to monitor the presence of all viral pathogens in the environment. The unreliability of bacterial model microorganisms led to the search for alternatives, and several bacteriophage groups appeared as promising candidates, among them somatic coliphages, F+ specific (male-specific), RNA bacteriophages and Bacteroides fragilis bacteriophages, all of them with available ISO (International Standardization Office) procedures for their detection in water.

Exhaustive studies are nevertheless still required to ascertain the validity of a candidate indicator in a given scenario. In the end we should probably give up our hopes of finding a "universal" indicator for viruses, applicable to all situations, and resign ourselves to the use of particular indicator, index, or model microorganisms for specific purposes.

Disinfection is an important treatment barrier between consumers and illness, however, current virus disinfection and/or removal practices often fail to adequately eliminate pathogenic viruses. Data on the stability of viruses relies mostly on bench-scale studies performed with available cell-adapted strains. A long-standing barrier to conduct actual field studies to evaluate the environmental behaviour of human enteric viruses is precisely the impossibility to introduce pathogens in the environment.

As model systems, recombinant surrogates are perfectly adequate for field studies of microbial tracking, since they may be produced in extremely high numbers (several milligram amounts). Additionally, their noninfectious nature makes them completely harmless and suitable to be used in scenarios where the use of actual viruses is hampered by the impossibility to introduce potential pathogens into drinking water treatment plants, shellfish growing waters, or selected foodstuffs. The use of recombinant virus-like particles may provide the tools for the systematic validation of virus removal practices in actual situations where pathogenic agents can not be introduced. Although bacteriophages and other microorganisms of

the fecal flora have been used as models of virus behaviour, from the strictly structural point of view, there is no better surrogate of an actual virus pathogen than a non-infectious virus-like particle of the same virus.

2.3 Viruses in Shellfish

Sam Bark and Kathleen Henshilwood
CEFAS Weymouth Laboratory, Weymouth, Dorset, UK

Previous research work, both in the UK and elsewhere in Europe, has developed methods for detection of Norovirus (NV) and hepatitis A virus (HAV), in shellfish. NV is the virus normally associated with human gastro-enteritis following shellfish consumption. A variety of methods have been published but no consensus method has yet emerged. However, all published methods rely on the polymerase chain reaction (PCR) for amplification of the low levels of virus found in environmentally contaminated shellfish to a detectable threshold. It is clear from the many reports now published that these methods do work for detection of NV and HAV in shellfish. However, it is also clear that a significant amount of work remains to be undertaken before these methods can be adopted into routine regulatory use. Regulations include a clause stating that the European Commission will introduce proposals for a viral standard with 3 years of the implementation of the regulations

It is now known that the Norovirus group contains a wide diversity of virus strains of related but differing sequence. However, such strain sequence information for the NV group has only become available comparatively recently. Although databases for clinical isolates are now quite extensive new strains continue to be recognised on occasion. The design of PCR primers against such a diversity of strains presents a difficult challenge. However, PCR primer design is a fundamentally important component of any PCR based assay. Workers in the field have proposed a variety of NV primer and probe combinations but no international consensus has yet emerged. Currently therefore, workers in the field use a wide variety of PCR primers to detect NVs in human clinical samples, shellfish and other matrices. Generally PCR products are detected by agarose gel electrophoresis followed by confirmation through probe blotting or sequencing. Assay formats in use include single round PCR and nested PCR to enhance assay sensitivity. The lack of standardisation in current approaches is a major problem for the development of a legislative standard for viruses in shellfish.

Real Time. PCR (TaqMan) chemistry is very well standardised and has significant advantages of speed, robustness, and labour costs. Significantly it incorporates a probe confirmation step into a one-tube single stage assay. Real-time PCR is also capable of providing quantitative data,

which may prove significant for the interpretation of field data for NVs in shellfish. However, design of 'catch-all' TaqMan primer/probes for NVs is a major challenge. It would be a major step forward to have real-time PCR assays available to cover the full range of NV strains. This would address both the standardisation, confirmation, and (potentially) the quantitation issues in one go. This presentation will provide background and recent progress on the development and use of a real time PCR method for detecting NVs in shellfish.

2.4 Enteric viruses in other foods, survival, methodology.

Soizick Le Guyader
Microbiology Laboratory IFREMER, Nantes, France

Noroviruses (NoVs, formerly Norwalk-like viruses) are the most common agents of outbreaks of acute gastroenteritis, and their transmission via contaminated food is increasingly recognized. Various types of food have been implicated in outbreaks: shellfish, vegetables, fruits, delicatessen foods, and bakery products. Because these viruses are resistant to environmental degradation, most chemical treatment processes, and are not altered by freezing, they can contaminate all types of food products either by sewage contamination during growth or by food handlers during harvest and packaging. Contamination could occur anywhere from the field to the table, and food handler hygiene is an important parameter throughout. A simple contact with soiled hands is sufficient for contamination with high levels of virus, and the common enteric viruses can stay infectious for quite a long time, even under modified atmosphere or after disinfection. Finally, the dose required for infection may be as low as a few particles for noroviruses. Combined, these factors provide a clear explanation for the great transmissibility of noroviruses and other non-enveloped enteric RNA viruses, such as hepatitis A virus, via food.

At present, diagnosis of such outbreaks relies mostly on epidemiological investigations, coupled with identification of the causative pathogen in persons with health complaints following consumption and, occasionally, in food handlers thought to be the source of infection. Final confirmation by detection of the pathogens in food still remains a challenge for various reasons. Firstly, few methods were developed for detection of viruses in foods and limitations exist to isolate and detect viruses in complex food matrixes. Secondly, most of the viral food-borne outbreaks are caused by noroviruses. These RNA viruses, which cannot be propagated in cell culture, are antigenically very diverse making the use of immunological methods difficult, and these methods are probably not sensitive enough to detect viruses in foods. Moreover, the genetic diversity of

these viruses has made the selection of a consensus primer set, and hence the use of RT-PCR more difficult.

However, methodologies for the direct detection of Norovirus in food are improving and recently, sensitive methods have been described for analysis of foods other than shellfish. All these methods rely on molecular detection of the agent and several factors such as extraction efficiency controls, avoidance of contamination during the PCR process, optimal RT-PCR conditions, or adequate primer sets, have to be taken into account for reliable and sensitive results to be achieved.

Some studies were done as part of an European project to identify common source food-borne virus outbreaks in Europe. It illustrates that it may be possible to develop sensitive methods for virus detection in food. Analysis of food samples in an outbreak investigation will help to evaluate the true role of food in the transmission of epidemic viral gastroenteritis. Whether viral analysis of food samples is of practical value in confirmation of foodborne outbreaks will require their application. The adaptation of protocols for different food matrices (e.g. fruits, liquids, meat products, etc) will be needed prior to their widespread use. When these aspects have been addressed, analysis of food samples for viral pathogens may become more feasible and provide valuable information. For example, recombinant noroviruses have been identified, and it is possible that multiple strain contamination of food or water that result in co-infections with more than one strain of norovirus may facilitate the emergence of such recombinant viruses.

Sensitive methods for the detection of noroviruses in various sources of infection, combined with powerful tools for genetic analysis, are crucial to improve our understanding of the evolution of these viruses. It is also important to better analyze the modes of transmission, how viruses are circulating between countries and to link variations between different outbreaks to genetic information about both hosts and viruses.

2.5 Zoonotic aspects of food and water borne enteric viruses

Wim H.M. van der Poel
Animal Sciences Group, Wageningen University Research, Lelystad, The Netherlands

Key words: Calicivirus, Sapovirus, Norovirus, Rotavirus, Hepatitis E virus, swine, cattle, gastro-enteritis, NV, SV, HEV

2.5.1. Introduction

Of all food and water borne enteric viruses some are clearly zoonotic and some others may have zoonotic potential. Moreover recent research indicates an increasing importance of zoonotic food or water borne viruses. In general all relatively resistant faecal-orally transmitted viruses can be implicated in food- or waterborne gastro-intestinal disease. Group A rotaviruses (family Reoviridae) are the most common cause of gastro-enteritis in young children and young animals. Several genotypes like G3, G6, G8 are shared between humans and animals, but direct transmissions among different animal species and between humans and animals species are not really observed. However, the increased number of reports of new human rotavirus genotypes, which are more commonly found in animals, suggest the possibility of interspecies transmission or genetic reassortment of rotavirus strains.

Noroviruses (NVs), formerly known as Norwalk-like viruses (NLVs), family Caliciviridae are the most common cause of acute non-bacterial gastro-enteritis in humans, and within the same virus family the Sapoviruses (SVs), formerly known as Saporo-like viruses (SLVs), are also associated with acute gastro-enteritis in humans. Noroviruses can be detected in swine and are present in cattle, but so far viruses genetically identical to bovine or porcine NVs have not been found in humans. Recently, a porcine enteric calicivirus (PEC), closely related to Sapovirus, has been identified, raising public health concerns about potential cross-species transmission. Zoonotic transmission has not been proven, but cattle and swine may be reservoir of NVs or SVs. Hepatitis E virus (HEV), a non-enveloped RNA virus, provisionally classified into a separate family of HEV-like viruses, is prevalent in humans as well as a number of animal species. Hepatitis E virus (HEV) is endemic in much of the developing world, and clinical illness resembles other forms of acute viral hepatitis, with fever and jaundice as the most common symptoms. The most important route of transmission is faecal-orally, and disease outbreaks are often associated with contaminated drinking water or bad hygienic conditions. To investigate if rotaviruses, noroviruses, sapoviruses, or hepatitis E viruses are commonly present in animals, and may be related to the strains found in humans, faecal samples of several animal species were assayed by reverse transcription-polymerase chain reaction amplification (RT-PCR). Detected sequences were genetically and phylogenetically analysed, to find out if these strains may have zoonotic potential.

2.5.2. Material and methods

Pooled and individual faecal samples, collected from cattle, swine, chicken and horses, from different regions of the Netherlands were assayed by reverse transcription-polymerase chain reaction amplification (RT-PCR),

as described (Van der Poel et al., 2000), using sets of primers specific for the detection of the strains found in humans (Gentsch et al., 1992: Gouvea et al., 1994; Jiang et al., 1999; Vinjé et al., 2000). RT-PCR products were cloned and sequenced and the obtained sequences were compared with genotypes detected in human or known to infect humans. Detected strains were molecularly typed and phylogenetically analysed.

2.5.3. Results

For rotavirus typing, a total of 110 human stool samples were successfully P and G genotyped by RT-PCR. All strains belonged to the main human rotavirus genotypes G1-4, G9 and P[4], P[6], P[8], P[9]) and P[8]G1 was predominant. 5.5% belonged to the G9 genotype. Eleven percent of all P[8] genotypes could only be genotyped by a recently published modified primer. Rotavirus positive faecal samples from 28 calf herds were genotyped by DNA sequencing. Genotypes G6 and G10 predominated; G6 was detected in 22 (78.6%) and G10 in 16 (57.1%) of the rotavirus positive calf herds. In 12 (42.9%) calf herds we found mixed infections. Genotype G8 was not found. Bovine rotaviruses G6 were divided in 3 clusters UK-like, VMRI-29-like and Hun4-like.

Sapovirus sequences were detected by RT-PCR in 28 of the pig farm specimens (25%). Norovirus RNA was detected in 33 of the specimens from veal calf farms (44%) and in 2 specimen from swine farms (2.0%), whereas samples from chicken farms and dairy cow herds were negative. By electron microscopy (EM), particles with the typical NV morphology were found in one veal calf farm sample. All NV sequences from calves were tightly clustered and were phylogenetically related to NVgenogroup I, whereas the swine viruses were phylogenetically related to NV genogroup II. Complete ORF2 sequences were obtained from 2 calf NVs and phylogenetic analyses clearly showed that these strains belong to a new cluster (GIII/2) within the proposed genogroup III NV. Overall, genetic variation between strains as determined by sequence analysis of the P1/P2 region of the capsid was limited to 14.6 percent. Our data indicate that NVs circulating in calves most likely are genetically distinct from NVs in humans.

Sapovirus sequences were only detected in swine and were clustered in two groups. The porcine enteric caliciviruses (PEC) that have been reported before and a second group designated as swine enteric caliciviruses (SWEC). Both of these clusters were phylogenetically related to human sapoviruses (Fig 1.). By RT-PCR testing of swine faecal samples we found a high prevalence of HEV in the Netherlands More than 22% of the pig farms were infected. HEVs from swine in the Netherlands were clustered in at least two groups, together with European and American isolates from swine as well as humans.

2.5.4. Discussion

Rotaviruses are generally species-specific, but cross-species transmission is possible, as has been demonstrated experimentally. Several case studies have indicated infection of humans by animal rotaviruses. Comparison of genetic sequences of human and animal rotaviruses often reveals close identity, but this was not demonstrated in the study of strains of animal and human origin in the Netherlands. The close phylogenetic relationship of NVs, SVs and HEVs with sequences of these viruses originating from humans that was found in the Netherlands, indicates that animals may be reservoir hosts of these viruses and could be a source of food and water borne enteric virus infections in humans. For caliciviruses it is known that these can be transmitted via food and water. In the case of HEV it is unclear how the virus may be transmitted from animals to humans, and it should be elucidated if food borne or water borne transmission are important. Unlike rotaviruses, so far Caliciviruses or hepeviruses identical to animal viruses have not been found in humans and therefore direct zoonotic transmission has not been proven. Nevertheless, for Caliciviruses, hepeviruses as well as rotaviruses there may be a continual input of strains or sequences into the human population from animal populations. Therefore the study of the strains of these viruses found in humans and animals should be continued to reveal which animal strains or sequences may enter the human population and may constitute a public health hazard.

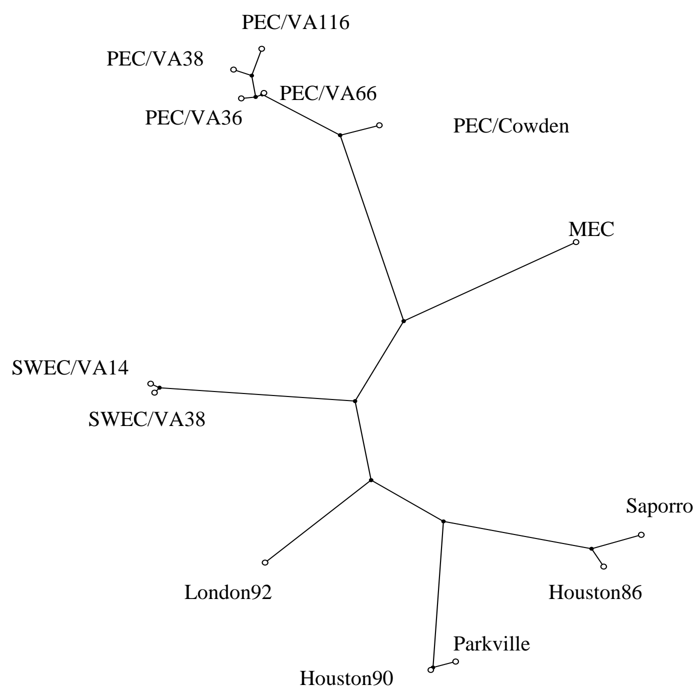

Fig 1. Maximum likelyhood Phylogram comparing polymerase (ORF1) gene sequences (145 nt) of porcine enteric caliciviruses (PECs and SWECs), mink enteric calicivirus (MEC) and human Sapoviruses (BioNumerics version 3.1, Applied Maths, Kortrijk, Belgium).

2.6 Identifying the sources of fecal contamination in water and shellfish using male-specific coliphages

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Numerous human pathogens (e.g., Vibrio cholera, Salmonella typhi, Giardia lamblia, Cryptosporidium parvum, Norovirus and Hepatitis A virus) are spread by fecal contamination of water. Due to difficulties in the detection, identification, and enumeration of specific human pathogens in water samples, indicator organisms are used to assess the potential for the presence of pathogens due to fecal contamination. In addition, information on the human and non-human origin of fecal pollution is important to enable appropriate management of the impacted waters. Traditional (fecal coliforms, E. coli) and alternative indicator microorganisms have been used for many years to assess and predict the presence of fecal pollution in water. In recent years, there has been increased interest in the use of male-specific or F+coliphages as indicators for microbial inputs to source waters and shellfish and the use of sero- or genotyping of these coliphages for microbial source tracking (MST).

There are six major families of coliphages, four of which (Siphoviridae, Podoviridae, Myoviridae and Microviridae) are somatic. Somatic coliphages infect their hosts through receptors on host cell walls. The other two families (Leviviridae, or FRNAs, and Inoviridae, or FDNAs) are male-specific, meaning they infect their hosts through via attachment to surface pili structures. Somatic and F+ coliphages have been consistently isolated from domestic, hospital and slaughterhouse wastewaters in concentrations between 106 and 1010 pfu/L, although concentrations can be lower during periods of heavy rainfall. Coliphages can also be found in treated wastewaters, which is indicative of the ability of viruses to survive treatment processes where bacterial indicators typically cannot. Both coliphages and enteric viruses appear to follow parallel seasonal patterns with higher levels during the colder months. FRNA coliphages appear to be consistently present in fecally polluted waters and do not appear to be present in non-fecally polluted waters.

The family Leviviridae contains two genera (Levivirus and Allolevivirus) and three unclassified groups (a, b, and c). The four coding regions of members of the Alloleviviruses are oriented in one single reading frame, while those of the Leviviruses are located in different reading frames that vary depending on the group. Based upon serological cross-reactivity, replicase template activity, and phylogenetic analysis, Levivirus and Alloleviviruses each contain distinct subgroups. The genus Levivirus contains group I and group II phages whereas the genus Allolevivirus contains subgroup III and IV phages. Based on currently available data, group II and III FRNA phages are mainly found in environments

influenced by human waste whereas group I and IV are mostly associated with animal pollution. However, as exceptions have been documented, this distinction is not absolute. Because antisera for serotyping of FRNA phages are not readily available and may give inconclusive results, genotyping of FRNA using direct nucleic acid hybridization has been successfully used in may field studies. Recent research involving genetic characterization of FRNA strains isolated from different sources demonstrated that most viruses grouped with the known prototype strains, but also identified viruses belonging to a potential novel genetic group. In addition, group III isolates from certain hog lagoons have been shown to form a separate subgroup showing that refined subtyping might be advantageous for MST.

This presentation will give an overview of the latest developments on the genetic characterization of FRNA and FDNA coliphages and their use in identifying human or animal fecal pollution.

3. Nordic activities of enteric viruses in food and water

This chapter contains Nordic research activities on enteric viruses in food and water. It includes abstracts of the presentations given at the workshop by the Nordic participants. The related presentations are shown in appendix D.

3.1 Calicivirus in food- and waterborne outbreaks in Sweden 2000–2004.

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Over the last 10 years norovirus, Caliciviridae, has emerged as the most common cause of foodborne gastroenteritis, and is at present the most common pathogen causing foodborne outbreaks in Sweden. Outbreaks with norovirus normally give rise to a greater number of cases than those caused by other foodborne pathogens.

Laboratory diagnosis of human calicivirus infections at the Swedish Institute for Infectious Disease Control, are based upon electron microscopy (EM). EM has the advantage of being a catch-all method but needs a high density of virus. In addition, all foodborne outbreaks were analysed by RT-PCR and characterised by sequencing or RLB (Reverse Line Blot). Two sets of primer pairs that amplify a 326bp fragment of the polymerase gene were used for the RT-PCR analysis (JV12A, JV13B, JV12Y, JV13I)(1, 2). However, when no virus was detected by the polymerase gene RT-PCR, primers targeting the capsid gene were used (COG2/ G2SKR, G1SKF/G1SKR, CapD/CapC, CapB/CapA)(3, 4).

During 2000–2003 on average 18 reported foodborne outbreaks per year (12–28 per year) were reported with approximately 800 cases (331–1212) each year; in total 74 outbreaks with 3183 cases. (This is to be compared with foodborne Salmonella that caused 39 outbreaks with 759 cases during the same period.) In comparison to the total number of calicivirus outbreaks in general, where there is a clear seasonal distribution with the highest prevalence in January through March, the food- and waterborne outbreaks were evenly distributed throughout the year. A small decrease could be observed during late spring and summer months.

Calicivirus was detected by EM in 39 (90%) foodborne outbreaks, while our routine RT-PCR failed to detect norovirus in six. Five of these outbreaks were later confirmed by the capsid gene RT-PCR. At least one sample from each outbreak was sequenced. The majority of samples from 2002 were genotyped by RLB, as well as by sequencing. These results showed a wide diversity of virus variants, which is in contrast to institutional settings, where one single strain dominated during the same time period. The new variant Grimsby, that completely dominated the European norovirus infections during the winter 2002–2003, did not have the same impact on foodborne outbreaks. During 2002, genogroup 2 strains were the most prevalent, but in as many as 48 percent genogroup 1 virus was found. All but two outbreaks in 2003, and 80% of the outbreaks this year (2004) were caused by genogroup 2 strains. No connection was found between a certain type of food and the genotype in patient samples. In addition to norovirus, two foodborne outbreaks were caused by sapovirus.

In approximately 68% of the outbreaks a suspected food vehicle was incriminated. There are few food items where norovirus has been demonstrated. We have detected virus twice in water and once in raspberries (5, 6). In theses cases, the corresponding genotype was detected among the patients. The difficulty to detect norovirus in food makes the epidemiological investigations, as well as the detection of norovirus in patients, more important than in other foodborne outbreaks. In cases when virus was detected in food, the preceding finding of virus in samples from patients was of great help in the development of a functional RT-PCR method.

The most implicated food in Sweden in norovirus outbreaks is uncooked frozen berries, served in pastries or as a cold sauce. Indirect contamination of food via an infected food handler is more likely when the food requires extensive handling, e.g. salads, tarts and cold cuts. The virus may also spread through drinking-water, raw shellfish and vegetables contaminated by sewage. During 2000–2004 seven waterborne outbreaks with 920 cases were investigated. The causes have often been leakage of drainage or lake water contaminating drinking water. Traditionally outbreaks around Christmas (buffet) and following New Years Eve (oysters) are reported. A large proportion of the outbreaks (44%), originate from restaurants, hotels and canteens, while only 7% are reported from households. For the majority of outbreaks there is no known contributing factor. Outbreaks with a contributing factor (33%) are usually associated with sick kitchen staff.

During the last years we have found several outbreaks, where norovirus was detected by EM but not detected by our diagnostic RT-PCR. The diversity among foodborne norovirus outbreaks demands broader working PCRs and emphasizes the importance of surveillance of circulating strains in the society in order to identify new strains. More information

and strict food hygiene rules for food handlers are needed to prevent foodborne outbreaks.

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3.2 Noroviruses in Finland

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Noroviruses (NVs) comprised the most important virus group that caused gastroenteritis epidemics in Finland in years 1998–2002. This finding is based on the viruses found from patient stool samples that were sent from epidemic situations to the Department of Virology, HUCS Laboratory Diagnostics in Helsinki. The samples were screened for rota- and adenoviruses by EM and RT-PCR was performed in search for noro- and astroviruses. Two or more samples per epidemic were obtained from 416 outbreaks out of which 252 (60.6%) were caused by noroviruses. During that time period only four epidemics were caused by astroviruses and one by rotaviruses. The children's samples that were sent to EM investigation only as a single sample are excluded in these numbers.

The number of norovirus epidemics ranged between 28 and 102 per year, the highest frequency occurring in year 2002. Norovirus epidemics occurred most often in hospitals or nurseries, but also frequently in restaurants, institutions like schools, spas etc. Genogroup II viruses outnumbered (86.5%) those of genogroup I viruses. Most of the established genotypes were found in Finland as well as a couple of sequences that did not group to them. The most common genotype was GII.4 (Bristol-like NVs); 68 out of 142 strains, from which a part of the polymerase nucleotide sequence was determined, belonged to this genotype. As many as 16

different amplicon sequences were detected during the five years within this genotype. One was the new variant that appeared in June 2002 for the first time in Finland and then replaced all the other variants with the exception of the so called intermediate strain for the rest of the year. This strain was very common in hospitals in year 2002 as was the GII.1 Hawaii-like genotype in year 2000. A strain shift was also very clear when the emerging GIIb genotype appeared in year 2001 and dominated during that epidemic season. The most common genogroup I genotype was GI.3 (Birmingham) that caused in total 17 epidemics. This genotype, however, never predominated but the epidemics were spread along the study period. The occurrence of genotype GII.7 (Leeds) increased in years 2001 and 2002 as compared to its earlier prevalence.

Several berry-related NV outbreaks occurred, mostly concerning raspberries. In a school epidemic in year 2002, NV genotype GI.3 was found in patients and the same genotype GI.3 and another genotype GIIb were isolated from the berry mix. The amplicon sequences were identical in GI.3 NV from patients and berries. In January 2001, oysters were found to contain two NV genotypes, GI.2 and GII.1. At the same month an oyster outbreak occurred with GIIb NVs in patient samples.

During the period 2000–2004 seven waterborne epidemics could be confirmed by norovirus findings from water and patient samples. In all cases the virus amplicons from both sources were identical in a particular epidemic. Four of these epidemics were caused by GII.4 genotype, once the new variant was the causative agent. Twice genogroup I virus GI.3 was isolated. The most recent waterborne outbreak represented genotype GIIb.

3.3 The status of viral food-infections in Iceland Jan 2000 – May 2004

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Until the year 2000 we had hardly any known outbreaks linked to noroviral infections in Iceland. Since then we have had some.

The first confirmed outbreak was in the end of the year 2000. Then 27 people from 4 companies got sick. Fecal samples were collected from 5 persons and calicivirus was confirmed from 4. The source was canapés that the people had eaten from the same catering service. No food samples were inspected.

The second outbreak was in April 2001 were 25 persons from the same company dining in the same canteen got sick. Fecal samples were collected from 2 patients, one of them calicivirus positive.

The third possible outbreak was in the end of July and beginning of August 2001. Then 117 foreign tourists from different tourist offices got sick and the only connection was that all stayed at the same hotel in the Nordic part of the country. Eight food samples were collected and researched for bacterial infection without any results. No fecal samples were collected as no one got hospitalized.

The year 2002 we had three outbreaks. In May we got 3 people calici positive after a small lunch meeting. The chef had story of illness. At the end of June and beginning of July 21 persons who all stayed at the same fishing lodge got sick. Fecal samples were collected from 4 patients and 3 were calicivirus positive. The source was unknown. About the same time another outbreak was reported not far from there in an area of summer-houses and camping. Sample was collected from one patient and was calicivirus positive. The source was unknown.

Last year there were no reports on outbreaks related to food poisoning. But since October 2002 we have had an enormous increase in viral hospital infections. Large calicivirus outbreaks were reported from hospitals and elderly homes.

3.4 Detection of Enteric Viruses in Shellfish from the Norwegian Coast

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Common blue mussels (Mytilus edulis) and horse mussels (Modiolus modiolus) obtained from various harvesting and commercial production sites along the Norwegian coast, were screened for the presence of norovirus by a real-time RT nested PCR, and for possible indicators of faecal contamination: f-specific RNA bacteriophages (f-RNA phages) by plaque assay and human adenoviruses by nested PCR. The aim was to obtain information relevant for assessing the risk of transmission of enteric viruses by shellfish, and to investigate the potential of various indicator viruses in routine screening. Noroviruses were detected in 6.8% of the samples, and the indicators in: 23.8% (f-RNA phages) and 18.6% (adenoviruses). A seasonal variation was observed for f-RNA phages and adenoviruses, with more positive samples in the winter. A positive correlation was found between f-RNA phages and noroviruses. However, f-RNA phages were present in only 43% of the norovirus positive samples. The results show that mussels from the Norwegian coast can constitute a risk of infection with enteric viruses, and that routine testing of samples may be justified. Various options for screening are presented.

3.5 Foodborne viral outbreaks in Denmark

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The summering of food borne outbreaks caused by enteric viruses is based on yearly reports of outbreaks of food borne diseases published by The Danish Veterinary and Food Administration. Included in these reports are outbreaks with well-founded evidence that a specific foodstuff or a specific meal has caused the illness. Enteric viral food borne outbreaks are here accounted if 1) clinical samples or incriminated foodstuff have been lab confirmed, 2) the case control studies compile with the criteria of Kaplan's or 3) if the only lack of Kaplan's is the missing clinical test of bacterial agents but kitchen staff has showed ongoing or recent story of enteric viral symptoms before food handling.

Before 2000, salmonellosis was the cause of the majority of involved persons in food borne outbreaks, which in 1999 exceeded 400 (37%) cases in 14 (25%) outbreaks. Since then and until end of 2003, the remarkably decrease in Salmonella food borne infected people has given space for enteric viruses to be the leading agents for persons involved in food borne outbreaks. During 1999–2003 40 (21%) food borne outbreaks with 1256 (38%) persons involved were reported to be due to enteric viruses; in average 31 cases pr outbreak. While 15 of these outbreaks were lab-confirmed to be caused by norovirus, rotavirus was lab-confirmed to be the incriminated agent in one outbreak and leaves 40% of the virus caused food borne outbreaks lab-confirmed.

In 50% of the outbreaks the incriminated food are traced back to restaurants, pizzerias and other takeaways, while only 7% have had its origin in private homes or parties (in between are catering, cantinas and supermarkets). A suspected food item was stipulated in approximately 50% of the outbreaks and that has been pizza, burger, grilled food, sandwich, wedding cake, and oysters (eaten in connection to New Years Eve). The common meals of the rest of the outbreaks have been 3-dinner meals or buffets. A limited amount of incriminated food items has been available for viral detection. Among 8 tested samples 4 of 5 oyster batches, which have caused independent outbreaks, have been confirmed norovirus positive. In some outbreaks infected meals-participants and secondary infections are thought to be the reason for the high number of affected people and maybe not the particularly food item.

3.6 Comparison of methods for detection of norovirus in spiked oysters.

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Abstract

In the absence of culture methods for noroviruses, detection in foods relies on molecular detection by Reverse Transcription –Polymerase Chain Reaction (RT-PCT) on extracted viral RNA. We have tested four RNA extraction protocols for their sensitivities and robustness to detect norovirus in spiked oysters. One method seemed to be more sensitive than the others using RT seminested-PCR. Not much difference was observed between the four methods by a RT single-PCR approach.

Introduction

It is well documented that outbreaks of viral disease related to consumption of bivalve shellfish harvested in faecal-polluted areas occur (Le Guyader et al., 1996, Lees 2000). Since oysters can filter 10–24 litres of water per hour - pathogens can be concentrated in the meat and give rise to human infections. In Europe, the pathogens of main concern are the gastrointestinal viruses like norovirus, which are known to cause outbreaks involving a large number of people, and Hepatitis A virus, which is known for its severe symptoms (Lees 2000).

Identification of enteric viruses from oysters implicated in gastroenteritis outbreaks can be difficult due to insufficient viral recovery and presence of PCR inhibitors. Several extraction procedures of viral nucleic acid from oysters have been described but few comparisons of their performance have been done. A typical protocol contains the following steps; homogenisation of tissue and viral elution, extraction and concentration of virus, RNA extraction and finally amplification of virus by the use of RT-PCR. In each of these steps there is a broad variation of procedures used by different laboratories, which calls for international consensus. During the implementation of methods for detection of virus in oysters, we evaluated different protocols for extraction of viral RNA to be used in the following RT-PCR detection. Thus the objective of the present study was to identify the most appropriate method for detection of norovirus (NoV) in oysters with respect to 1) sensitivity, 2) robustness and 3) ease of handling. This was done by comparing four different protocols for their ability to detect norovirus in spiked oysters.

Materials and methods

Oysters from the Danish market, Crasosstrea gigas and Ostrea edulis harvested in France and Denmark respectively, were collected during the winter 2002–2003. Digestive glands were cut out, mixed and aliquoted in 1.5 g portions and stored at -80°C for further use. The 1.5 g portions were later spiked with 100 µl of 10-fold serial dilutions (range 10^{-1} to 10^{-4}) of stool samples earlier found positive for NoV and determined by sequencing to contain a genogroup II strain. The positive stool samples were kindly provided by Dr. Blenda Böttiger, Statens Serum Institute, Denmark. Before spiking with virus, all batches were examined and found negative for natural content of NoV using the method described by Le Guyader et al., 1996 for RNA extraction and the genotype specific RT-seminested PCR primers G1SKR/COG1F + G1SKR/G1SKF and G2SKR/COG2F + G2SKR/G2SKF for NoV amplification and detection (Kojima et al., 2002 and Nishida et al., 2003).

The four selected protocols for detection of virus in oysters are shown in Table 1. The four protocols A, B, C and D are essentially according to Le Guyader et al., 1996; De Roda Husman et al., 2004; Mullendore et al., 2002; and Beuret et al., 2002, respectively.

NoV amplification and detection was carried out by RT-PCR and using the generic primers JV13I/JV12Y targeting the RNA polymerase gene (Vinjé et al., 1996; and Vennema et al., 2002) and the genogroup II specific RT-seminested PCR primers G2SKR/COG2F + G2SKR/G2SKF in the capsid region (Kojima et al., 2002; and Nishida et al., 2003). RT-PCR reactions were run on a PTC-225 Peltier Thermal Cycler, MJ Research. The level of recovery was based on the highest virus dilution giving a clear positive signal after RT-PCR, which was visualized on a 2% ethidium bromide stained agarose gel and subsequent confirmed by sequencing (MWG-Biotech, Germany). All four protocols were randomly applied on triplicates of oyster tissue seeded with the different dilutions (10^{-2} – 10^{-4}) of NoV positive stool samples.

Results and discussions

All the methods could be implemented in the laboratory and detect NoV from 1.5 g of seeded oyster tissues see table 2. Method B was found to be the most sensitive method. Thus using method B we were able to detect NoV in all 3 samples spiked with NoV positive stool samples diluted 10^{-4} when the RT seminested – PCR primers was applied for detection of viral RNA. RT seminested – PCR is known to have an advantage compared to ordinary RT- PCR when samples contain high amounts of inhibitors. None of the other methods were able to detect NoV in this dilution. In addition method B was faster and easier to handle than any of the other methods in this study. On the other hand, little difference was observed between the four methods when a single RT-PCR approach was used for

detection of NoV. This may indicate that the better sensitivity obtained with method B is more likely due to a good viral recovery rather than to removal of inhibitors. A possible improvement of inhibitor removal could be to include a chloroform extraction step prior to the use of the RNeasy Plant and Funky kit. Method A and B are chosen for further use in surveillance of naturally contaminated molluscan shellfish from Danish harvest areas.

Table 1: Principles of the four RNA extraction methods for virus-oyster processing used for recovery of norovirus in the present study.

	Methods			
Processing steps	A (Le guyader et al., 1996)	B (De Roda Husman et al., unpubl)	C (Mullendore et al., 2002)	D (Beuret et al., 2002)
Homogenisation/ virus elution	Glycine pH = 9.5	Tissue disruption in a Mixer Mill (Bead Beater) with Lysisbuffer, RTL and zirconia beads, followed by centrifugation.	Acid absorption of viral particles to tissue solids. Glycine elution pH=7.5. Re-elution of pellet in threonine. PEG precipitation.	Glycine elution pH=7.5. Re-elution of pellet in threonine. PEG precipitation.
Virus extraction	Chloroform/Butanol + CatFloc-T.	-	PBS+Chloroform Chloroform-phase re-extracted with threonine.	PBS+Chloroform.
Concentration	PEG precipitation	-	PEG precipitation followed by GITC wash	PEG precipitation followed by GITC wash
RNA extraction	Proteinase K + phnol/chloroform Ethanol precipitation, Cethyltriaminborat wash, Ethanol precipitation	Rneasy® MiniKit, Plant and Fungi, QIAgen.	QIAamp Viral RNA Mini Kit, QIAgen.	QIAamp Viral RNA Mini Kit, QIAgen.

Table 2: Detection of norovirus from seeded oyster tissue processed by four different RNA extractions methods, A, B, C and D. For detection of virus two conventional detection formats RT-PCR and RT-seminested PCR were used.

Detection format	**Artificially contaminated oyster tissue (ml stool/1.5 g oyster)**	**RNA processing methods [1)]** A	B	C	D
RT- PCR	1×10^{-2}	2/3	3/3	2/3	3/3
	1×10^{-3}	2/3	3/3	2/3	1/3
	1×10^{-4}	0/3	1/3	0/3	0/3
RT-seminested PCR	1×10^{-2}	3/3	3/3	2/3	2/3
	1×10^{-3}	3/3	3/3	1/3	1/3
	1×10^{-4}	0/3	3/3	0/3	0/3

1: See table 1 for keys.

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Conclusions and recommendations

The epidemiology of enteric viruses and the procedures for the detection of these microorganisms have been discussed in the Nordic co-operation at several occasions. There is an agreement that the outbreak investigations presently rely mainly on epidemiological tools and the detection and characterisation of viruses in clinical samples from humans. However methods for their detection in food and environmental samples have shown important benefits during the investigations where incriminated outbreak sources have been available for analysis, and where epidemiological data were scare. The presentations and discussions during this workshop verify that it is beneficial and of most importance to continue the work on method development.

In this workshop, the current state of knowledge on Food and waterborne viruses was presented. In the following sections, highlights from the workshop discussions are stated with focus on the remarks, conclusions and recommendations related to the areas discussed.

- All Nordic countries have implemented PCR for the routine detection of norovirus in clinical samples and the activities for virus detection in food and water are increasing.
- All Nordic countries have designated National Reference laboratories on bacteriological and virological contamination of bivalve molluscs.
- *Norovirus* has been identified as a major cause of diarrhoeal illness in all Nordic countries.
- Some of the main risk factors for viral foodborne infections include the consumption of raw or undercooked shellfish, contaminated drinking water and desserts made of raw frozen berries.
- The difficulties to detect norovirus in food make the epidemiological investigations, as well as the detection of norovirus in patients, more important than in other foodborne outbreaks.
- Case-control studies are carried out in many countries but they need to be be more frequent and more extensive to be of importance in identifying risk factors and risk behaviours.
- Sensitive and quantitative detection methods as well as genotyping methods need to be further developed and validated.
- The role of animal and environmental factors in transmitting enteric viruses needs to be further studied.
- Information accumulated in the Nordic countries should be used in a joint formal risk assessment.

- The risk of transmission of food borne enteric viruses through the lack of general hygiene measures needs to be communicated to food handlers and consumers.
- It is of great importance to maintain/strengthen the Nordic Network created through previous years workshops on viruses in food and water. This networking facilitates new and more concrete internordic network and research project collaborations.
- Evaluation of detection methods for viruses in foods is an ongoing activity in several Nordic countries. Collaboration and a joint Nordic funding would strengthen these activities. In addition this could improve our influence in the International working groups connected to the development of method standards (CEN/TC275 WG6/TAG4) and shellfish monitoring systems (Reference Laboratory network for microbiological monitoring of shellfish), where problems are discussed and decisions are taken.
- The Nordic shellfish industry is among the largest in Europe and it is presently in a huge growth. It is therefore of great importance that we participate in the research activities of virus detection in foods in order to participate in the decision making processes regarding systems for virus monitoring including selection of methods for detection of viruses in foods.

In conclusion, available methods for norovirsus detection and identification are often sufficient to identify the different species involved in human infections and to find match between victims and sources among ill kitchen staff. However, the methods cannot quantify the number of noroviruses and are most often limited when we wish to find a match between isolates from patients and the possible food sources of infection. In relation to detection and genogroup typing, RT-PCR or realtime RT-PCR is the preferred method, while sequencing is the preferred and only tool for genotyping and source tracking. The development of quantitative methods for analysis is in progress along with the exploration of realtime PCR detection. Still, continued efforts need to be given to the extraction procedures for viral RNA in both food and water matrices before candidate methods can be reached for standardisation purposes. However, a single detailed, internationally validated protocol may not be suitable for all food or water matrices. More collaboration in the field of method development is therefore necessary.

Appendix 1: Workshop Programme

June the 1st and 2nd 2004.

Tune Landboskole, Greve, Denmark.

Monday the 31st of May

17^{00} - 19^{00}	The school is open for Check in. Sandwich, Fruits and Coffee will be available.

Tuesday the 1st of June, 9:00 – 17:30.

9^{00} - 10^{00}	Check in, coffee and bread
10^{00} – 10^{15}	Welcome and introduction by *Chairman Birgit Nørrung*, Head of Dept. of Microbiological Food Safety, DFVF, DK.
10^{15} – 11^{00}	Food and waterborne viruses. Health aspects, epidemiology and preventative measures. *Blenda Böttiger, SSI, Denmark*
11^{00} – 11^{45}	Enteric viruses in water. *Albert Bosch, UB, Spain*
11^{45} – 13^{00}	Lunch break.

Chairman Jeffrey Hoorfar, DFVF, DK

13^{00} – 13^{45}	Enteric viruses in bivalve molluscs, elimination, methodology and faecal indicators. *Sam Bark*, CEFAS, UK

$13^{45} – 14^{30}$ Enteric viruses in other foods, survival, methodology. *Soizick Le Guyader*, IFREMER, FR.

$14^{30} – 15^{00}$ Coffee break.

$15^{00} – 15^{45}$ Zoonotic aspects of food and waterborne enteric viruses. *Wim van der Poel,* RIVM, NL.

$15^{45} – 16^{30}$ Identifying the source of fecal contamination in water and shellfish using male-specific coliphages. *Jan Vinjé, CDC, USA.*

$16^{30} – 17^{30}$ Nordic presentations (max. 20 minutes per country).

FI: Norovises in Finland. *Leena Maunula.*

SE: Calicivirus in food- and waterborne outbreaks in Sweden 2000-2004. *Marika Hjertquist and Maria Lysén.*

IS: The status of viral food-infections in Iceland. *Sigrún Guðnadóttir.*

NO: Detection of enteric viruses in shellfish from the Norwegian coast. *Mette Myrmel.*

DK: Food borne virus in Denmark and NMR funded project “Comparison of viral RNA extraction procedures in oysters”. *Anna Charlotte Schultz.*

19^{00} Dinner

Wednesday the 2nd of June, 9:30 – 16:00.

Chairman: Jan Vinjé, CDC, USA

$8^{30} – 10^{00}$ Nordic presentations continued from yesterday completed with a short summary.

$10^{00} – 10^{30}$ Coffee break

$10^{30} – 11^{15}$ Literature study on detection methods for enteric viruses in water.

Anders Dalsgaard, KVL, DK

11:15 – 11:30	CEN/TC 275 WG6 TAG 4. Info on method standardization for virus detection in food. *Wim van der Poel, RIVM, NL*
11:30 – 12:00	Introduction to working groups.
12:00 – 13:00	Lunch break
13:00 – 15:00	Working group discussions (Coffee available from 14:30)
15:00 – 15:45	Results of group discussions
15:45 – 16:00	Summary of discussions, progress and recommendations.

End of meeting

Appendix 2: Workshop Participants

Country	Name	Institute
Spain	Albert Bosch abosch@ub.edu	Enteric Virus laboratory, Department of Microbio-logy, University of Barcelona, Diagonal 645, DK-08028 Barcelona, Spain
France	Soizick Le Guyader Soizick.Le.Guyader@ifremer.fr	Microbiology Laboratory IFREMER, Rue de L 'ile D'Yeu, BP1049-4400 Nantes, France
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The Nederland	Wim van der Poel Wim.vanderPoel@wur.nl	Division of Infectious Diseases, Animal Sciences Group, Wageningen University Research, P.O. Box 65, 8200 AB Lelystad, The Nederlands
United States	Jan Vinjé JVinje@cdc.gov	Head, Calicivirus Laboratory DVRD/NCID. Centers for Disease Control and Prevention (CDC). 1600 Clifton Road NE. Atlanta, GA 30333
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Denmark	Blenda Böttiger BBO@ssi.dk Christina Johnsen cjo@ssi.dk	Staten Serum Institute (SSI), Virus Diagnostics Laboratory, Artillerivej 5, DK-2300 Copenhagen S, Denmark
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