Identifying International Foodborne Norovirus Outbreak Events

A Combined Epidemiological and Molecular Approach

Linda P. B. Verhoef

ISBN 978-90-6464-453-5

The research described in this thesis was conducted at the National Institute for Public Health and the Environment (RIVM), The Netherlands, and was funded by the Dutch Food and Consumer Product Safety Authority (nVWA), the European Commission, DG Research Quality of Life Program, under the 6th Framework (EVENT; SP22-CT-2004-502571) and SG SANCO (DIVINE-net, 2003213).

Cover photo by Rob Pastoor (www.robpastoor.com)

Lay-out by Linda Verhoef

Printed by Ponsen & Looijen B.V., Wageningen.

Printing of this thesis was supported by the RIVM, Erasmus University (EUR) and nVWA.

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A Combined Epidemiological and Molecular Approach

De identificatie van internationale voedselgerelateerde norovirus uitbraken

Een gecombineerde epidemiologische en moleculaire aanpak

Proefschrift

ter verkrijging van de graad van doctor aan de

Erasmus Universiteit Rotterdam

op gezag van de rector magnificus

Prof.dr. H.G. Schmidt

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

donderdag 7 april 2011 om 15:30 uur door:

Linda Paulina Bernardina Verhoef-Pastoor

geboren te Arnhem

Promotiecommissie

Promotor:

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Prof.dr. R.A.M. Fouchier

Copromotor:

Dr. W. van Pelt

Voor mijn vader

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General Introduction

Chapter 1

Public health institutes like Municipal Health Services (GGD'en) and the Food and Consumer Safety Authority in the Netherlands (nVWA) are obliged to undertake action when food forms a threat to public health. Foodborne infections in general, including norovirus (NoV) infections, are notifiable if two or more persons are related to each other, with suspicion for food or water as a source (http://www.rivm.nl/cib/infectieziekten-A-Z/infectieziekten/ calicivirus/index.jsp). Unfortunately, outbreaks or sporadic infections caused by viruses in food are difficult to recognize as such. In addition, a foodborne outbreak may not be a mere local or national problem but an international problem. This is especially true for the diffuse foodborne outbreaks, or geographically disseminated outbreaks, in which a single batch of food may be consumed in several regions or countries simultaneously. Such outbreaks are likely to occur when foods are contaminated early in the food supply chain, i.e. during cultivation or production, and then exported to several regions or countries. Moreover, such products may be kept frozen with a long shelf life, causing outbreaks over a prolonged period of time. Given social and economical developments like the globalization of the food market, the trend towards reuse of wastewater for irrigation, and given the fact that quality control criteria for food do not suffice for viruses in food, such diffuse outbreaks are likely to increasingly occur with ready-to-eat food products in the near future.

In the absence of assays with detection levels low enough to detect viruses in a range of naturally contaminated food products, the main challenge for foodborne viral illness containment lies in the furnishing of proof and causality. We considered that this may need to go beyond the epidemiological and virological methods currently available. Given a rich dataset of combined epidemiological and virological data available for noroviruses, this pathogen was used to explore possibilities of development of new methods combining epidemiological and virological data. This thesis is the result of the search for new methods assisting the identification of diffuse foodborne norovirus outbreaks.

Virology

Viral Characteristics of Relevance for Food Safety

Viruses can be considered the smallest of all self-replicating organisms. Historically, they are characterized by their ability to pass through filters that are suitable for retaining the smallest bacteria. Unlike bacteria, viruses do not have a metabolism of their own, and they are obliged to invade host cells and parasitize the sub cellular machinery [128]. There is a large variety in structures of viruses. The genome can consist of DNA or RNA, in double- or single-stranded form. The virus structure can vary from simple spheres to more complex structures. In general, the more complex structures are less resistant to environmental influences. For example, viruses can be either non-enveloped or enveloped by a membrane consisting of a lipid structure. The enveloped viruses are more susceptible to detergents like soap or alcohol, since the infectivity of the virus will be disturbed if the membrane is affected. Foodborne transmission is a possible route of fecal-oral transmission. Viruses that follow the fecal-oral route have evolutionary been adapted to survive the low pH in the stomach and the high pH in the bowel. Thereby, these viruses are often environmentally stable, especially the non-enveloped and small round structured viruses [18].

History of Noroviruses

Norovirus (NoV) infection was already described as a cause of illness in 1929 [368], although at that time it could not yet be identified as such. In 1972 it was first visualized, using electron microscopy (EM). Small round virus particles [158] were found in stool samples from an outbreak of gastroenteritis in **Nor**walk, **O**hio in 1968, and the virus was initially named Norwalk virus. Later, its name was shortened to norovirus. In 1990, cloning and sequencing of this Norwalk prototype strain enabled the development of molecular methods for detection of norovirus [365]. Since then, noroviruses (NoVs) were increasingly detected as a cause of illness, and nowadays noroviruses are also known as the 'winter vomiting disease' or the 'cruise ship virus' and considered the most prevalent causes of viral gastroenteritis. To date, there is no *in vitro* propagation system for noroviruses [85], which leads to complications in detection of low viral doses, and in proving viability.

Characteristics of Noroviruses

Noroviruses are non-enveloped, single-stranded, positive-sense RNA viruses, consisting of a ~7.6-kb genome in three open reading frames (ORFs) (Figure 1). The first ORF (ORF1) encodes a polypeptide whose processing yields multiple non-structural proteins; ORF2 encodes the viral capsid protein (VP1); and ORF3 encodes a minor structural protein (VP2). On the basis of the first isolated norovirus strain (Hu/NV/I/Norwalk/1968/US) ORF1 comprises 5214 nucleotides, ORF2 1590 nucleotides, and ORF3 636 nucleotides.

Figure 1. Schematic representation of the norovirus genome. Numbering is based on the GII.4 genome. The different domains in ORF2 are depicted.

NoVs have been classified as a genus in the family of *Caliciviridae*. Noroviruses are genetically and antigenically highly variable. The diversity among NoVs is maintained through the accumulation of point mutations associated with the error-prone nature of RNA replication [119, 307]. Some mutations may be silent, whereas others can lead to amino acid changes. Currently five norovirus genogroups (GI-V) have been described, of which 3 (GI, GII and GIV) are known to cause illness in humans [120]. GII is also found in swine [224] and GIV in lions [222], whereas GIII mainly infects cattle [224], and GV mice. The noroviruses infecting humans are considered exclusively human pathogens. Even though a huge number of NoV infections and outbreaks have been studied, there is no proof of zoonotic transmission and/or an animal reservoir for human infections. The five genogroups are subdivided into at least 40 genotypes based on their amino acid capsid sequence [120, 174, 186]. Although genogroup I genotypes are relatively more frequently seen in foodborne outbreaks, most impact in recent years has been caused by viruses from a single genotype in GII, the GII.4, causing illness worldwide [304, 308]. Within the genotype II.4 new variants have emerged every other year since 2002 [1, 181, 205, 304, 341]. Unlike some other viruses, norovirus genotypes do not show a distinct geographical distribution. The emergence of some new variant strains seems to have occurred globally, whereas other variants caused outbreaks in only a limited geographic area [308].

Clinical Features of Norovirus Infections

Norovirus infection is mostly described to be self-limiting. Clinical symptoms of norovirus infection in humans comprise acute onset of vomiting and (watery) diarrhea. Onset of symptoms is typically 12-72 hours after exposure, and symptoms may last 1 to 3 days although longer recovery times have been described for elderly, children and immuno-compromised patients [73, 210, 211, 242, 307, 321]. Back in 1982, the clinical symptoms have resulted in the criteria as proposed by Kaplan to be indicative of norovirus outbreaks: an incubation period of 15 to 50 hours, vomiting in >50% of all cases, and/or diarrhea, average duration of symptoms 12-60 hours, high attack rate and stool samples that test negative for bacterial pathogens [159]. Norovirus is detected in fecal samples collected during the acute phase, but detection may be possible in stools collected up to 3 weeks after recovery. Recently, some chronic shedders have been identified that have not been able to clear the infection due to their immuno-compromised state [307]. Norovirus is highly infectious and affects all age groups. Although commonly known as a mild disease, recent studies revealed that norovirus infections may be potentially life-threatening for the elderly and that significant higher mortality may be associated with norovirus infections [20, 281]. Asymptomatic shedding has been repeatedly reported for NoV [104, 115] complicating, amongst others, diagnosis.

Detection of Noroviruses in Patients and Food

Methods for the diagnosis of norovirus infection in humans differ widely between countries [204, 348]. NoVs are usually detected in stool, particularly in outbreaks [71, 87]. Over the last decades, methods for detection have shifted from the gold standard of electron microscopy to molecular techniques with consequent broader applicability, and potential for higher sensitivity [204] and international comparison of strains [348]. Routine laboratories may currently not be able to perform these methods, but this situation is changing rapidly. In Europe, most countries have at least one specialized laboratory capable of detecting NoVs in stool [204]. Real–time reverse transcription polymerase chain reaction (RT-PCR) amplification techniques allow for (semi-)quantitative analysis [217] and have been described for NoV [81, 138, 139, 156, 157, 232, 253, 273, 296, 330]. Initially, RT-PCR techniques were optimized using a limited range of strains, resulting in sensitive detection but with a narrow diagnostic range. The past decades, the RT-PCR techniques have been developed towards more generic and broader detection methods. Most assays target conserved regions which also allow for genotyping of strains [173]. For monitoring trends at the level of genotypes, resolution of sequence-based typing does not need to be very high, and this can be achieved by sequencing a relatively conserved genomic fragment [308]. However, the identification of epidemiologically linked patients requires sequence typing at a much higher resolution level, since a single mutation may be informative of a link between cases or outbreaks. Thus, for linking patients the detection of a different genomic region may be required.

For virus detection in food products, sample preparation is challenging due to the low doses and unequal distribution of noroviruses in foods. To date, detection of noroviruses in food remains complicated for foods other than shellfish, although methods have been developed and validated [18, 23]. For shellfish, which are filter-feeders, higher amounts of noroviruses are present as a result of filtering the water they grow in. Detection of noroviruses in food showing the presence of norovirus RNA is still confined predominantly to specialized laboratories. The inability to prove the viability of the detected viruses in foods

complicates conclusions towards the causal relationship between food consumption and illness, and towards responsibility with respect to food safety.

Epidemiology

The History of Surveillance

John Snow was one of the founders of epidemiology. In 1848 he ventilated the hypothesis that cholera was spread by contaminated water [241]. He traced the water used in the houses affected by cholera to a specific water pump in Broad Street, London. Later observations indicated 700 deaths within a 250-yard radius of the Broad Street pump, and use of its water was strongly correlated with death from cholera. Thus 'geographical epidemiology' began, although it took some years before Snow's observations were generally accepted. At that time, microbiological proof was not yet available to confirm his hypothesis. From that moment on, monitoring of infectious diseases occurred in the frame of public health and patient care, and gradually became more systematic as surveillance. Surveillance has been defined as "*systematic ongoing collection, collation and analysis of data and the timely dissemination of information to those who need to know so that action can be taken*" (www.who.int). Thus, such data collections are typically applied for preventive and control measures [328]. The field of infectious diseases profits from surveillance, since early recognition of outbreaks enables timely measures, source tracing and thereby prevention of newly infected cases. To enable timely detection of outbreaks, the complexity of surveillance systems need to balance the time needed for data-entry and the minimum information needed for timely recognition. For these reasons, surveillance systems commonly need simple structures demanding minimal load from data providers, enabling ongoing and timely reporting of information.

The History and Characteristics of the FBVE Network Database

In Europe, the Foodborne Viruses in Europe (FBVE) network was established as a surveillance system for norovirus outbreaks in 1999 [183, 204]. The FBVE network is a collaboration between epidemiologists and virologists in 13 countries (Figure 2), and their aim was to monitor trends in NoV outbreaks, in order to study the diversity of the noroviruses involved, and to combine this data to detect international outbreaks with a common foodborne source enabling source tracing. Although the name FBVE suggests a foodborne focus, the network has actually collected data on outbreaks from all modes of transmission to obtain a comprehensive overview of viral background activity in the community. Knowledge of this background diversity enables to separate an outbreak or epidemic strain from the endemic prevalence of strains. The FBVE network has developed combined epidemiological and virological outbreak reporting with aggregation and sharing of data through a joint web-based database. In this database, a dataset with epidemiological information is registered, along with data on the diagnostic methods used, as well as outbreak strain sequences. The FBVE network was one of the pioneers collecting both laboratory and epidemiological data on norovirus outbreaks, providing us the opportunity to explore such data for the development of new methods which can assist in the detection of diffuse foodborne outbreaks.

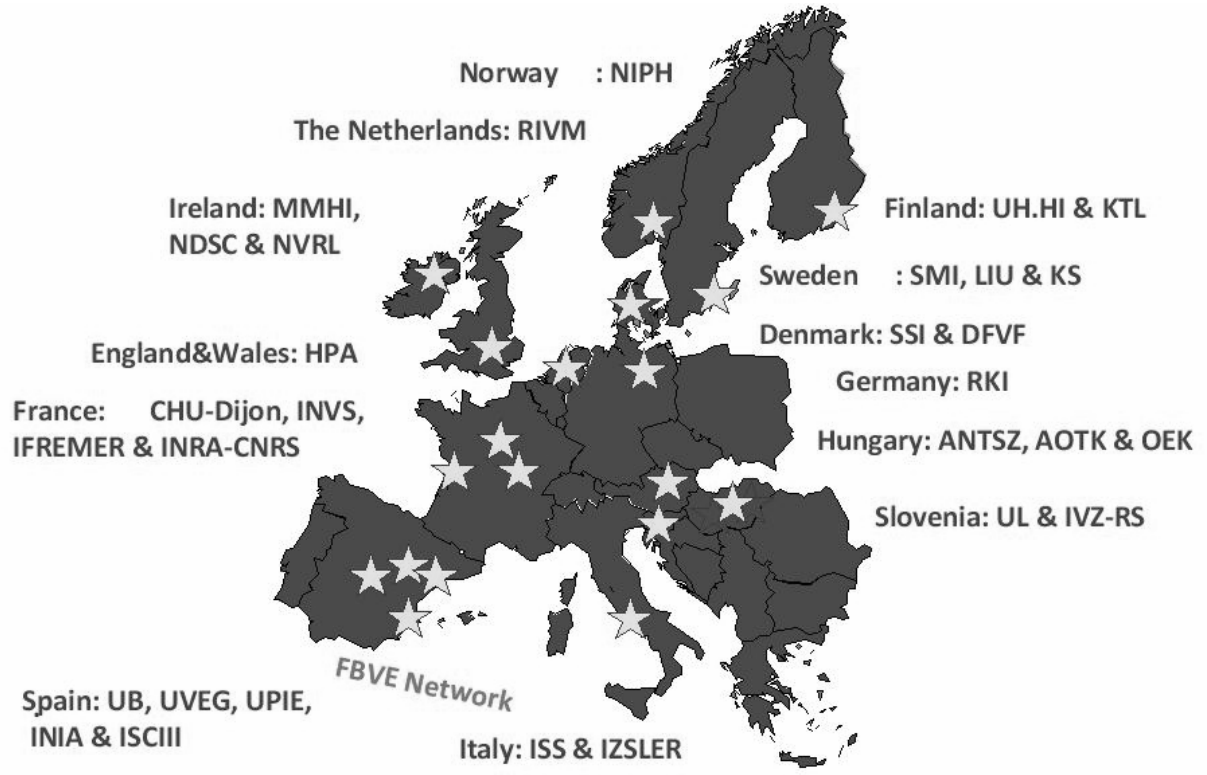

Figure 2. The Foodborne Viruses in Europe network.

The Burden of Norovirus Illness

Few studies have looked at the incidence and health impact of NoV infection in population-based studies. The most extensive data are from the Intestinal Infectious Disease (IID) study in the UK [73, 355] and the Sensor study in the Netherlands. In both population-based studies a randomized sample of the community participated in cohort studies. Viruses were the most frequently identified causes of community acquired gastroenteritis, with estimates of NoV incidence of 31 per 1000 person years in the Netherlands, and 13 per 1000 person years in the UK. This difference may partly result from the different methods used for virus detection: de Wit et al. used RT-PCR whereas the study in the UK employed the far less sensitive electron microscopy. While in the UK a second IID study was recently finalized, the SENSOR study is to date the basis for estimating the burden of norovirus illness in the Netherlands: the burden of norovirus was estimated to be 450 DALYs with an estimated incidence of 470,000 cases (2.9% of the Dutch population), costing society 25 million euros in 2004 [162]. For comparison, in the same population, the burden of disease for Salmonella and Campylobacter were estimated to be 8.8 million euros and 19.6 million euros respectively.

Transmission of Noroviruses

Noroviruses are easily transmitted following the fecal-oral route, either directly or indirectly via persons, contaminated surfaces, water or food [86]. Norovirus has a great potential for person-to-person transmission as no more than 10–100 particles are required for infection, and particles are shed in very high quantities [78, 214]. In theory, 1 mg of feces behind a nail would be sufficient to infect 10.000 people. A recent study has estimated norovirus as the most infectious virus so far described [327]. Given the infectivity,

noroviruses are easily spread which is mostly recognized as person-to-person transmission, especially in closed settings like nursing homes [165], cruise ships [63] and child care centers [215]. Also, secondary person-to-person spread of noroviruses after primary introduction by, for example, food, is common. Therefore, outbreaks caused by foodborne introduction of norovirus may not be recognized as such. The following two major routes for norovirus contamination of foods can be identified: i) via human sewage and faeces and ii). infected food handlers [18]. Following these main routes, foods can become contaminated from farm to fork, i.e. during cultivation, irrigation, harvesting, production, processing, preparation and serving. Many of the documented outbreaks of foodborne viral illness have been linked to contamination of prepared ready-to-eat food, which are commonly contaminated during the preparation or serving phase by an infected foodhandler [252]. During production, the use of sewage-contaminated water for cultivation or irrigation or rinsing may be the main cause of contamination. Examples of involved risk foods are foods that are minimally processed before consumption, like bivalve mollusks and fresh or frozen produce [93, 353]. Transmission routes of noroviruses will be discussed extensively in this thesis.

Food Safety Perspectives

Because control of pathogens in the food chain requires hazard analysis of critical control points (HACCP) systems to be implemented and verification of measures taken, detection of the pathogen or indicator is considered an important step [143]. However, viral contamination of food is less likely to be recognized than bacterial contamination, since viruses do not grow in food [117], and foods acceptable by bacterial standards are not necessarily free from viral contamination. The widely held standard for microbial safety of food, i.e. the presence of less than the European legislation limit of 230 *Escherichia coli* per 100 g (ISO 16649-3), has very little correlation with the presence or absence of viruses [103]. For example, norovirus may be present in shellfish that meet the European Union *Escherichia coli* standard for human consumption [106]. For other foods no routine detection is performed and the prevalence of viral contamination is unclear. Data are limited to confirmation of viral presence in foods related to outbreaks [285, 352]. A negative test outcome does not necessarily indicate safety of the tested batch of food. Risk foods commonly seen in norovirus outbreaks are shellfish [24, 70, 79, 127, 164], soft fruits like raspberries [61, 93, 136, 177, 194, 227], and ready-to-eat foods like sandwiches [24, 115]. Although these products are often associated with outbreaks, other products may be risk foods as well, but may be missed. Source tracing and sampling are not necessarily part of surveillance or outbreak investigations, and food items may not be requested in a systematical way.

Given the globalization of the food market, a single batch of food is likely to be consumed in several countries simultaneously [155]. As some foods may be kept frozen for two years, a single batch of food may be consumed over a prolonged period of time. There are only limited guidelines for the quality of irrigation waters, and the hygienic standards during the production process vary between countries. In some regions of the world, irrigation waters can be contaminated with human sewage [18, 55, 319]. Consequently international viral outbreaks caused by a common foodborne source are likely to occur. This is especially true for outbreaks involving foods that were contaminated early in the food supply chain. Descriptions of geographically dispersed outbreaks [70, 116, 264, 310, 353] illustrate the difficulties of linking cases and food in space and time especially when frozen ready-to-eat foods are involved.

The Need for New Methods Combining Virology and Epidemiology

Proof for Action

Although epidemiological association with a foodborne source may be sufficient evidence to result in measures in some countries [3, 19], detection of the causative agent in food is the preferred situation for action in most countries including the Netherlands [259, 337]. A foodborne viral source can be identified by comparing viral 'fingerprints', i.e. the nucleotide (nt) sequences. This would enable measures like withdrawing the product from the market. The problem is that virus strain matching in food and patients is rarely succeeded for foods other than shellfish. Thus, establishment of (international) links between common source foodborne outbreaks relies mainly on detection of noroviruses in humans, and either descriptive or analytical epidemiological data like surveillance, case-control or cohort studies. The predictive power of the use of sequence data to confirm outbreaks depends on the availability of background data; as systematic norovirus surveillance is limited to few countries, this currently limits their use. However, the field is moving fast, and implementation of molecular methods in laboratories across the world underscores the promise of these methods.

Complications in Tracing Foods Involved in Outbreaks

Analytical studies are laborious and more likely to be performed for the larger outbreaks [247], or those receiving media attention [339, 342, 351]. Unfortunately, several of these studies illustrate the difficulty currently encountered in pinpointing a food product for viral outbreaks of gastroenteritis, even when foodborne transmission is suspected. For example, in a one-year intensified gastroenteritis outbreak surveillance study in the Netherlands [336], suspected food items were a broad range of products. For these products, leftovers were only available for 50% of the outbreaks and all tested negative. Foodborne transmission may also be difficult to disentangle from the secondary person-to-person transmission. This was illustrated in a recent cohort study where consumption of juice served in self-service tanks was associated with disease. Unfortunately, no food sampling was performed and the transmission route remained unresolved. This outbreak was probably caused by using the taps of juice served in large containers with a tap for self-service, due to environmental contamination through person-to-person transmission. Still the role of contaminated juice could not be ruled out. [351].

Need for New Methods

Public health authorities are by law obliged to report and implement measures to contain foodborne outbreaks. Such outbreaks may not be a national but an international problem. Given the globalization of the food market, a foodborne outbreak due to contamination of food during the production phase is likely to cross borders. Unfortunately, as described above, foodborne outbreaks at either the national or international level may be difficult to recognize as such. Clearly, the identification of foodborne viral outbreaks cannot rely on molecular testing only, and there is no possibility to perform an analytical study for each suspected foodborne outbreak. Thus, there is a need for combined molecular and basic epidemiological criteria to assist public health efforts in identifying diffuse (international) foodborne outbreaks. New methods and tools are needed to improve the recognition of such foodborne transmission. Given a rich dataset of combined epidemiological and virological data available for noroviruses, this pathogen can be used as an example.

Scope of this Thesis

The aim of this thesis was to develop methods for linking international foodborne viral outbreaks, caused by contamination of food early in the food chain in order to enable source tracing and recall of foods for secondary prevention.

Approach: To explore the database of the FBVE network to find whether combined analysis of virological and epidemiological outbreak-based norovirus surveillance data can supply these methods.

In **chapter 2** we give an overview of the FBVE data after a period of 5 years of combined laboratory and epidemiological data collection. This analysis showed that there are large differences in the numbers and rates of reported outbreaks per country, reflecting the differences in the focus and coverage of national surveillance systems. Spring and summer peaks indicated the emergence of genetically distinct variants within GII.4 across Europe and were followed by increased NoV activity. To determine whether the biennial emergence of new variants may be due to a common source event, further investigated was performed at the time of sudden increase in reports of cruise ship related norovirus outbreaks in 2006, which is described in **chapter 3**. During this investigation, detection of a point source was not possible, probably because secondary transmission had masked the connection between sources and outbreaks. This was the illustrated in **chapter 4** with a cohort study onboard one of these ships, during the second of successive outbreaks. These investigations illustrate the need for indicators of foodborne transmission. The FBVE database was therefore analyzed to identify virological and epidemiological indicators of food-relatedness of norovirus outbreaks. **In chapter 5** we present a validated predictive model which was the basis of a practical web-based tool to assist public health workers in limiting the number of outbreaks needing source tracing. It was found that genotype non-II.4 outbreaks were more often related to a foodborne source compared to II.4 outbreaks. This topic was investigated while increasing molecular detail in chapters 6 and 7. **In chapter 6**, we demonstrate that further specification into genotypes shows additional differences in the probable epidemiology of norovirus outbreaks. We identified two distinguishable genotype profiles enabling the separation of outbreaks due to contamination of food during the production phase from those probably contaminated through infected food handlers. In **chapter 7** we aimed to use strain variability among sequences to provide criteria to determine whether clusters of norovirus sequences may represent international common-source outbreaks, or 'outbreak events'. We estimated that international collaboration could have significantly increased the number of recognized international foodborne outbreaks. In **chapter 8** we show that the genomic regions obtained with commonly applied primers are not specific in the recognition of outbreak events, which may explain the difficulty in recognizing these events as single groups within the background diversity of strains. This analysis was a joint effort with researchers in the field of bioinformatics, and has provided a generic method for the identification of the informative genomic region for linking outbreaks. This method can be used by laboratory networks to evaluate their efforts of recognizing outbreak events as such. In **chapter 9** we describe the progress made in the field of viral foodborne diseases over the past decade, which is for a large part attributable to the progression made in norovirus research. Despite the progress made, the challenge remains in furnishing of proof and causality of a foodborne source of infection. In **chapter 10** we discuss the possibility of identifying international foodborne norovirus outbreaks, and how the here described new methods can contribute to source tracing and secondary prevention of foodborne norovirus illness.

An Analysis of Integrated Virological and Epidemiological Reports of Norovirus Outbreaks Collected within the FoodBorne Viruses in Europe Network from 1 July 2001 to 30 June 2006

Chapter 2

Annelies Kroneman, Linda Verhoef, John Harris, Harry Vennema, Erwin Duizer, Yvonne van Duynhoven, Jim Gray, Miren Itturiza, Blenda Böttiger, Gerhard Falkenhorst, Christina Johnson. Carl-Henrik von Bonsdorff, Leena Maunula, Markku Kuusi, Pierre Pothier, Anne Gallay, Eckart Schreier, Marina Höhne, Judith Koch, György Szücs, Gábor Reuter, Katalin Krisztalovics, Maureen Lynch, Paul McKeown, Barbara Foley, Suzie Coughlan, Franco M. Ruggeri, Ilaria di Bartolo, Kirsti Vainio, Elmira Isakbaeva, Mateja Poljsak-Prijatelj, Ada Hocevar Grom, Janet Zimsek Mijovski, Albert Bosch, Javier Buesa, Alicia Sanchez Fauquier, Gloria Hernandéz-Pezzi, Kjell-Olof Hedlund, and Marion Koopmans.

J Clin Microbiol 2008;46:2959-65

Abstract

The Foodborne Viruses in Europe network has developed integrated epidemiological and virological outbreak reporting with aggregation and sharing of data through a joint database. We analyzed data from reported outbreaks of norovirus (NoV)-caused gastroenteritis from 13 European countries (July 2001 to July 2006) for trends in time and indications of different epidemiology of genotypes and variants. Of the 13 countries participating in this surveillance network, 11 were capable of collecting integrated epidemiological and virological surveillance data and 10 countries reported outbreaks throughout the entire period. Large differences in the numbers and rates of reported outbreaks per country were observed, reflecting the differences in the focus and coverage of national surveillance systems. GII.4 strains predominated throughout the 5-year surveillance period, but the proportion of outbreaks associated with GII.4 rose remarkably during years in which NoV activity was particularly high. Spring and summer peaks indicated the emergence of genetically distinct variants within GII.4 across Europe and were followed by increased NoV activity during the 2002–2003 and 2004–2005 winter seasons. GII.4 viruses predominated in health care settings and in person-to-person transmission. The consecutive emergence of new GII.4 variants is highly indicative of immune-driven selection. Their predominance in health care settings suggests properties that facilitate transmission in settings with a high concentration of people such as higher virus loads in excreta or a higher incidence of vomiting. Understanding the mechanisms driving the changes in epidemiology and clinical impact of these rapidly evolving RNA viruses is essential to design effective intervention and prevention measures.

Introduction

Noroviruses (NoVs) are small, nonenveloped RNA viruses that are increasingly reported as causes of gastroenteritis across the world. Data from population-based studies suggest that NoVs are the most common cause of infectious gastroenteritis at the community level in developed countries. Little information is available about the role of NoV in gastroenteritis in developing countries [46, 105, 233]. While illness associated with NoVs is typically mild and self-limiting, their high incidence and transmissibility result in large numbers of outbreaks, for which NoV has become notorious. Outbreaks occur in people of all ages and are particularly common in health care settings or other institutions where transmission may be facilitated by crowding and possibly lower standards of hygiene [27, 109, 206]. Here, the impact of NoV may be more severe [226], and costs of controlling outbreaks may be high.

Recent events have suggested changes in the epidemiology of NoVs, when an unexpectedly high number of outbreaks on cruise ships signaled the start of a major epidemic in 2002 [205]. Similarly, the spring of 2006 witnessed numerous outbreaks on cruise liners across Europe [182], triggering a joint outbreak investigation by the European Centers for Disease Control and the Foodborne Viruses in Europe (FBVE) network to identify possible sources for these outbreaks and provide clues from detailed investigations on the basis of the joint data set [341]. Reports from other parts of the world have also suggested increased incidence of NoV outbreaks [1, 288].

NoVs are genetically highly variable and have been divided into five genogroups, which can differ as much as 40% with regard to the amino acid composition of the major capsid protein (VP1) [369]. Genogroups are further divided into genotypes, defined by strains with a higher level of homology across the VP1 (80%). An increasing number of genotypes are recognized as well as additional sublineages within genotypes [111, 137, 200, 305, 332]. GII strains, particularly GII.4, are found most commonly all over the world [27, 109, 305].

We analyzed the data submitted to a joint database for 24 institutes in 13 countries in Europe, to study the trends in NoV outbreak reporting and the distribution of circulating strains of NoVs identified between July 2001 and July 2006. We show that rapid evolution of NoV occurs all over Europe and speculate that emergence of new variants at regular intervals is associated with high levels of outbreak reporting.

Methods

The Network

The FBVE network was established in 1999 and is a collaboration between epidemiologists and virologists in 13 countries. A web-based database was established in 2001 by the network to provide a systematic collection of data on outbreaks of viral gastroenteritis in Europe [174]. A data set with epidemiological information was reported, along with data on the diagnostic methods used, as well as sequence information from outbreak strains. A description of the projects and an example of the outbreak questionnaire and the database design can be found via www.fbve.nl.

Definitions Used

A NoV outbreak was defined as an outbreak (two or more cases linked in place and time) of gastroenteritis with laboratory-confirmed NoV infection. Gastroenteritis was defined as two or more episodes of vomiting in a 12-h period and lasting at least 12 h and/or two or more loose stools in a 12-h period and lasting at least 12 h. Laboratory confirmation of a NoV outbreak means two or more of a minimum of five stool specimens obtained from persons in the acute phase of the illness testing positive. The NoV high season was defined as date of onset of the outbreak between 1 July year x and 30 June year x + 1.

Strain Characterization

The genotype of NoV was determined based on partial sequence analysis of the polymerase gene and/or capsid gene [119, 347]. Definitive assignment of a genotype in the database entries was performed by one molecular virologist from the coordinating team according to our publicly available typing system (www.rivm.nl/bnwww). The GII.4 strains were then subdivided into five variants, based on distinct phylogenetic clustering and unique motifs [306]. The variants were assigned as GII.4-year, based on the first year in which this clustering became apparent. Due to a lag in reporting and the need to have a number of sequences before a cluster can be recognized, this is often a year later than the actual first outbreak with this new variant.

Initially, all genotyping within the network was based on partial polymerase gene sequencing, but following developments in the diagnostics of NoV some countries have switched to genotyping based on partial capsid gene sequences. For this study, the polymerase and capsid genotypes were combined and grouped into three classes: GII.4, all other genogroup II strains, and all non-genogroup II strains. For part of the analyses the GII.4

Table 1. Outbreaks per season per country

	Value by season*											
	2001/2002				2002/2003				2003/2004			
Country †	N all (%)‡	rate §	N type (%)¶	N type + epi (%)#	N all (%)	rate	N type (%)	N type + epi (%)	N all (%)	rate	N type (%)	N type + epi (%)
DE	74 (8.7)	0.9	74 (15.7)	74 (21.8)	99 (8.8)	1.2	99 (22.1)	98 (27.4)	0	-	0	0
DK	21 (2.5)	3.9	1 (0.2)	1 (0.3)	5 (0.4)	0.9	2 (0.4)	1 (0.3)	4 (1.2)	0.7	0	0
ES	43 (5.0)	1.0	34 (7.2)	19 (5.6)	36 (3.2)	0.9	24 (5.4)	15 (4.2)	4 (1.2)	0.1	3 (2.6)	0
FI	55 (6.5)	10.6	45 (9.6)	9 (2.6)	118(10.5)	22.7	22 (4.9)	3 (0.8)	22 (6.6)	4.2	18 (15.4)	3 (4.5)
FR	9 (1.1)	0.1	7 (1.5)	5 (1.5)	13 (1.2)	0.2	11 (2.5)	11 (3.1)	9 (2.7)	0.1	8 (6.8)	4 (8.1)
EW	485 (56.9)	8.2	191(40.6)	133 (39.1)	563 (50.0)	9.5	60 (13.4)	42 (11.7)	192 (57.8)	3.2	11 (9.4)	0
HU	92 (10.8)	9.0	61 (13.0)	43 (12.6)	112 (9.9)	11.0	97 (21.7)	63 (17.6)	51 (15.4)	5.0	39 (33.3)	22 (33.3)
IE	0	-	-	-	0	-	-	-	0	-	-	-
IT	1 (0.1)	0.0	1(0.2)	0	2 (0.2)	0.0	2 (0.4)	1 (0.3)	1 (0.3)	0.0	0	0
NL	59 (6.9)	3.7	55 (11.7)	54 (15.9)	148 (13.1)	9.1	107 (23.9)	101 (28.2)	27 (8.1)	1.7	25 (21.4)	25 (37.9)
NO	0	-	-	-	0	-	-	-	0	-	-	-
SE	7 (0.8)	0.8	1 (0.2)	2 (0.6)	12 (1.1)	1.3	10 (2.2)	9 (2.5)	15 (4.5)	1.7	13 (11.1)	12 (18.2)
SL	6 (0.7)	3.0	0	0	19 (1.7)	9.5	14 (3.1)	14 (3.9)	7 (2.1)	3.5	0	0
Total	852 (100)	2.4	470 (100)	340 (100)	1127 (100)	3.5	448 (100)	358 (100)	332 (100)	1.6	117 (100)	66 (100)

*Date of onset between 1 July year x and 30 June year x + 1; † DE, Germany; DK, Denmark; ES, Spain; FI, Finland; FR, France; EW, England and Wales; HU, Hungary; IE, Ireland; IT, Italy; NL, The Netherlands; NO, Norway; SE, Sweden; SL, Slovenia; ‡ N all (%), number of outbreaks per country and % of all outbreaks of that season; § Rate, number of outbreaks per million inhabitants per country (population on 1 January of 2002 to 2006; source, Eurostat [http://epp.eurostat.ec.europa.eu]). Total rate per season is calculated using only the contributing countries within that season in the denominator; ¶ N type (%), number of outbreaks with typing data and % of all outbreaks with typing data; # N type + epi (%), number of outbreaks with typing data and known mode of transmission and setting and % of all outbreaks with typing data and known mode of transmission and setting.

Table 1 – *continued*

	Value by season*										
	2004/2005				2005/2006				whole period: 2001/2006		
Country†	N all (%)‡	rate§	N type (%)¶	N type + epi (%)#	N all (%)	rate	N type (%)	N type + epi (%)	N all (%)	N type (%)	N type + epi (%)
DE	1500 (64.1)	18.2	0	0	2135 (71.5)	25.9	0	0	3808 (49.9)	173 (9.4)	172 (13.2)
DK	7 (0.3)	1.3	3 (0.7)	3 (1.1)	18 (0.6)	3.3	17 (4.3)	16 (5.8)	55 (0.7)	23 (1.2)	21 (1.6)
ES	14 (0.6)	0.3	13 (3.1)	3 (1.1)	30 (1.0)	0.7	27 (6.8)	23 (8.4)	127 (1.7)	101 (5.5)	60 (4.6)
FI	54 (2.3)	10.3	31 (7.5)	5 (1.9)	44 (1.5)	8.4	16 (4.0)	9	293 (3.8)	132 7.1)	29 (2.2)
FR	26 (1.1)	0.4	26 (6.3)	18 (6.8)	31 (1.0)	0.5	31 (7.8)	24 (8.7)	88 (1.2)	83 (4.5)	62 (4.8)
EW	357 (15.3)	5.9	41 (9.9)	0	341 (11.4)	5.6	81 (20.4)	0	1938 (25.4)	384 (20.8)	175 (13.4)
HU	81 (3.5)	8.0	71 (17.1)	41 (15.4)	62 (2.1)	6.2	41 (10.3)	34 (12.4)	398 (5.2)	309 (16.7)	203 (15.6)
IE	81 (3.5)	19.7	68 (16.4)	67 (25.2)	128 (4.3)	30.4	44 (11.1)	44 (16.0)	209 (2.7)	112 (6.1)	111 (8.5)
IT	9 (0.4)	0.2	5 (1.2)	1 (0.4)	3 (0.1)	0.1	2 (0.5)	1 (0.4)	16 (0.2)	10 (0.5)	3 (0.2)
NL	168 (7.2)	10.3	132 (31.9)	104 (39.1)	120 (4.0)	7.3	101 (25.4)	89 (32.4)	522 (6.8)	420 (22.7)	373 (28.6)
NO	16 (0.7)	3.5	0	0	22 (0.7)	4.7	0	0	38 (0.5)	0	0
SE	11 (0.5)	1.2	10 (2.4)	10 (3.8)	26 (0.9)	2.9	20 (5.0)	20 (7.3)	71(0.9)	54 (2.9)	53 (4.1)
SL	16 (0.7)	8.0	14 (3.4)	14 5.3)	25 (0.8)	12.5	18 (4.5)	15 (5.5)	73 (1.0)	46 (2.5)	43 (3.3)
Total	2340 (100)	6.4	414 (100)	266 (100)	2985 (100)	8.2	398 (100)	275 (100)	7636 (100)	1847 (100)	1305 (100)

group was subdivided into the separate variants. If available, a strain was assigned to one of these classes or variants based on phylogenetic clustering of the polymerase sequence. When only the capsid sequence was determined, this was used for classification.

Recombination is common in NoV, and as a rule the recombination point is located at the open reading frame 1 (ORF1)/ORF2 overlap region, which is between the polymerase and capsid regions used in this study [42, 272]. This implies that results from polymerase-based genotyping and capsid-based genotyping cannot be combined without specifically addressing this issue. We concluded that combining the two typing methods using the above-described division into three classes was valid on the basis of an analysis of the subset of 264 entries in the data set used in this study which contained both polymerase and capsid sequences. This showed that only 2% (n = 5) of the strains would be assigned to another class when using capsid genotype.

Data Analysis

For this study, we selected all outbreaks with a diagnosis of NoV and date of onset between 1 July 2001 and 31 June 2006 from a download of the FBVE outbreak reporting database of 4 June 2007. The number of outbreaks each month and in each country was determined. Per analysis a subset of outbreaks was selected based on the availability of data. For these selections, the number of outbreaks by genotype and variant was determined in each month, by suspected mode of transmission and by setting. GII.4 strains were compared to GII non-4 and GI strains. Potential risk factors were determined from the minimum data set including month and year of onset of the outbreak, setting, and suspected mode of transmission, using multinomial logistic regression models. To limit the number of dummy variables in the model, potential risk factors were converted to binary variables. Variables significant during univariate analyses ($P < 0.05$) were included in a multivariate model. The variables remained in the multivariate model if P values were below 0.05 while using the backward selection procedure, when they were found to be confounding factors for other variables in the model (β changing at least 10%) or when they were effect modifiers (X^2 of the interaction term significant at $P < 0.05$).

Results

Representativeness of Data

For the period of 1 July 2001 to 30 June 2006, 7,637 NoV outbreaks were reported to the FBVE reporting database (Table 1). The rates (number of outbreaks as a proportion of the population size of each country) differed by country, ranging from 0.0/million in 2001-2002 and 2003-2004 in Italy, where only one outbreak was reported in both years, to 30.4/million in 2005-2006 for Ireland.

Two countries, Germany and England and Wales (these latter two being considered one country for reporting purposes), supplied 3,808 (49.9%) and 1,938 (25.4%) of all outbreaks in the data set, respectively. Ten countries—Denmark, Spain, Finland, France, England and Wales, Hungary, Italy, The Netherlands, Sweden, and Slovenia—reported outbreaks throughout the 5-year period. Twenty-four percent (1,847/ 7,637) of the reported outbreaks contained data on genotype (based on submitted sequences). Within this subset, 50% of the data were provided by three countries, England and Wales, Hungary, and The Netherlands, with 20.8%, 16.7% and 22.7%, respectively. Seven countries — Spain, Finland,

France, England and Wales, Hungary, The Netherlands, and Sweden — submitted outbreak reports including sequences throughout the complete period.

The subset of reports containing both genotyping and epidemiological background information on setting and mode of transmission was 1,305 records (17.1%). The data from large contributors were analyzed separately to look for possible biases in the global analyses in order to differentiate between national and international trends. These data are presented separately where relevant.

Trends in Overall Outbreak Reporting, 1 July 2001 to 30 June 2006

Monthly trends in outbreak reporting for the 10 countries which reported throughout the complete period showed clear winter seasonality (Figure 1). The size and month of the peak differed annually, with the largest number of outbreaks reported in the 2002–2003 period and remarkably low levels of reporting in the 2003–2004 period. The outbreaks from England and Wales are shown separately from those of the other countries because they show a slightly different pattern. In the years 2001–2002, 2002–2003, and 2005–2006 the peak in England and Wales was observed somewhat earlier than in the other countries, and increased reporting was observed in the spring of 2004 unlike in other countries. Off-seasonal outbreak activity was observed across Europe in the spring of 2002. The seasonal peak month in each country was determined for each season. No direction of spread across Europe could be identified visually this way (data not shown).

Trends in NoV Outbreaks by Genotype, 1 July 2001 to 30 June 2006

Outbreaks were grouped by genotype for the countries that submitted these data throughout the reporting period (Figure 2), showing that GII.4 strains were the predominant genotype within the 5 years under surveillance. In England and Wales the proportion of GII.4 outbreaks was lower throughout the entire period. The winter seasonality was clearest for outbreaks caused by GII.4 strains, and a weaker seasonal pattern was visible for other GII viruses. Within the GII non-4 strains mostly II.2, II.7, and II.b strains were found (data not shown; typing based only on polymerase sequences) with the 2001– 2002 peak of GII non-4 outbreaks mainly caused by II.b. II.b is the preliminary name for a distinct strain with a polymerase sequence that did not cluster with any known sequence globally. It was initially associated with oyster-associated outbreaks all linked to the same source and subsequently found in other settings [40]. The 2005–2006 peak was dominated by II.7 and II.b (based on polymerase typing; data not shown).

No seasonal pattern was observed for non-GII outbreaks. Univariate logistic regression analysis confirmed that GII.4 strains were more common between October and March than were non-GII strains (odds ratio [OR], 1.9; 95% confidence interval [CI], 1.2 to 3.1; P = 0.006). For other GII outbreaks this was also significant (OR, 1.8; 95% CI, 1.7 to 3.0; P = 0.0322), whereas non-GII outbreaks occurred throughout the year. A similar pattern was observed for individual countries, although this was not statistically significant due to low numbers. The 2002–2003 and the 2004–2005 winter peaks were almost exclusively caused by GII.4 NoV.

Trends for Individual GII.4 Variants, 1 July 2001 to 30 June 2006

Following initial observations on emergence of new variants within GII.4, we decided to study the trends in individual GII.4 variants. Plotting trends for individual GII.4 variants showed consecutive emergence and disappearance of new variants across Europe (Figure 2). In the 2001–2002 season the predominant II.4 variant was GII.4 1996, which had also

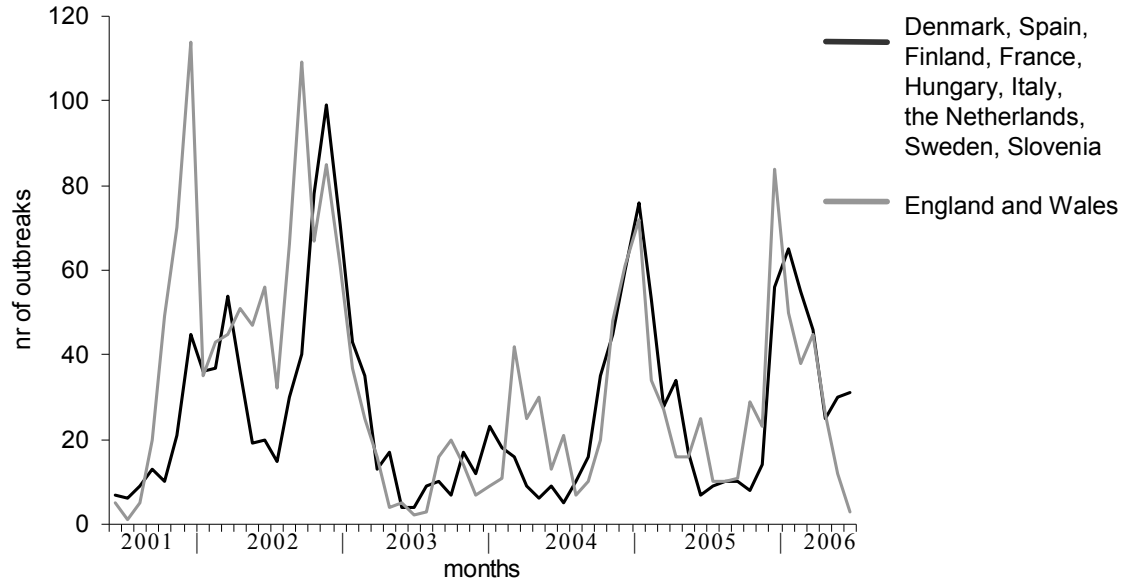

Figure 1. Number of reported outbreaks per month of onset for all countries reporting throughout the complete period.

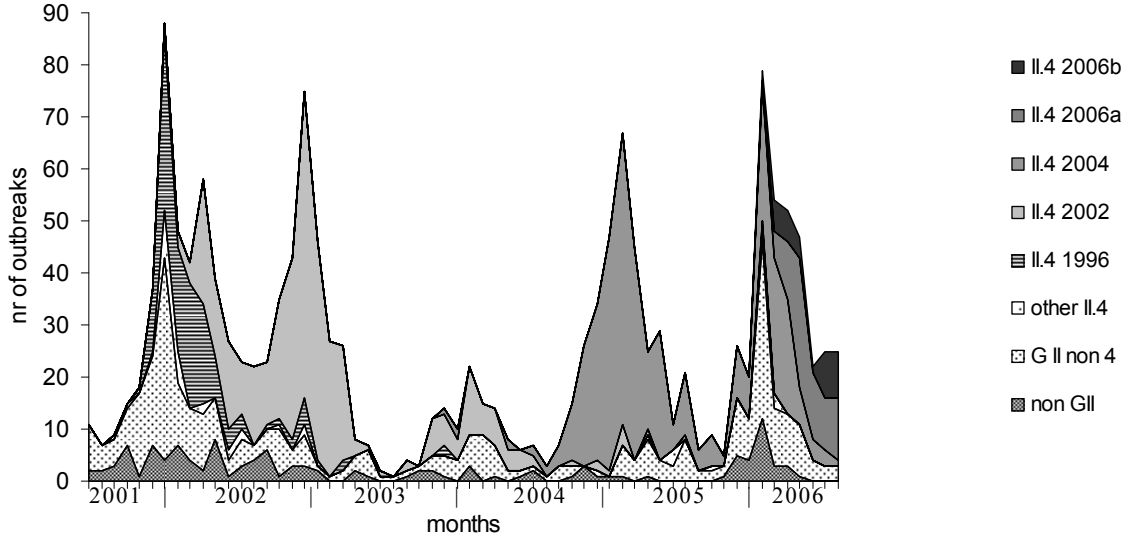

Figure 2. Number of outbreaks with genotypes and variants per month from Spain, Finland, France, England and Wales, Hungary, The Netherlands, and Sweden (the subset of countries which have outbreak reports with sequence data throughout the complete period).

been circulating in years prior to this survey [306]. In 2002–2003 and 2004–2005 the peaks were almost exclusively caused by a new variant which was first reported a few months earlier. In 2006, two distinct variants were seen cocirculating. The first report of variant GII.4 2002 in the database was from two outbreaks in January 2002 in residential institutions in The Netherlands, with person-to-person transmission. Subsequently this variant rapidly became predominant in the course of the year. The following season, 2003–2004, showed very low NoV activity, but the first outbreaks with the new II.4 2004 variant were seen cocirculating with the 2002 epidemic strain. In the 2004–2005 season this new variant had become predominant, with only a few outbreaks of II.4 2002. In the 2005–2006 season the 2002 variant was not detected anymore.

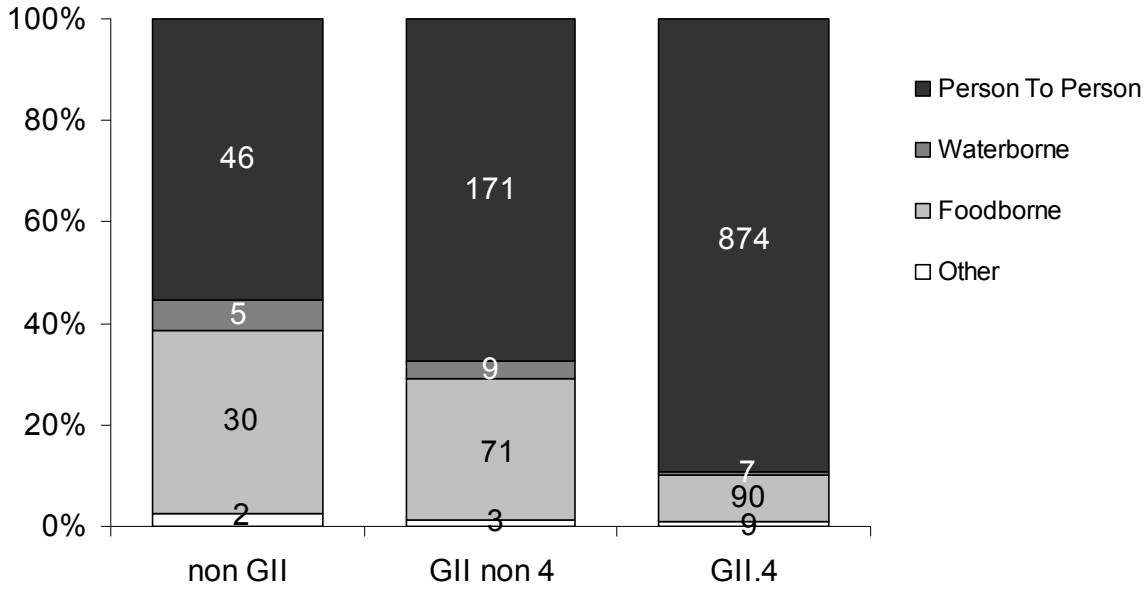

Figure 3. Suspected mode of transmission for all genotyped outbreaks with reported mode of transmission (n = 1,317).

Variant II.4 2004 was reported for the first time in an outbreak which took place in November 2003 in Finland with an unknown setting and mode of transmission. The first reports of variant II.4 2006a in the database were in two outbreaks in February 2006 in France and The Netherlands in residential institutions, with person-to-person transmission. Variant II.4 2006b was firstly reported in an outbreak in December 2005 in two residential institutions in Spain. Patterns for 2002 and 2004 variants were similar across the network. For the 2006 variants, some geographic differences were observed. In Hungary, only the 2006b variant was found during the study period. No geographical direction of spread could be deduced visually from the first outbreak or the peak month for the individual variants in the different countries (data not shown).

Modes of Transmission Overall and by Genotype

Of all outbreaks with a reported mode of transmission (n = 5,036), 88% (n = 4,429) were suspected to be person-to-person outbreaks, 10% (n = 506) were foodborne outbreaks, and 2% (n = 76) were waterborne outbreaks. For the subset of outbreaks with genotyping data (n= 1,317), again the main (suspected) mode of transmission (83%) was person to person (Figure 3). As Figure 3 shows, person-to-person outbreaks were relatively more often caused by GII.4 viruses and foodborne outbreaks were relatively more often caused by non-II genotypes. Univariate logistic regression analysis showed that person-to-person transmission was an independent risk factor for GII.4 outbreaks (OR, 6.3; 95% CI, 3.8 to 46.6; P < 0.001) but not other GII outbreaks (OR, 1.6; 95% CI, 1.0 to 2.6; P = 0.0736), compared to genogroup non-II outbreaks.

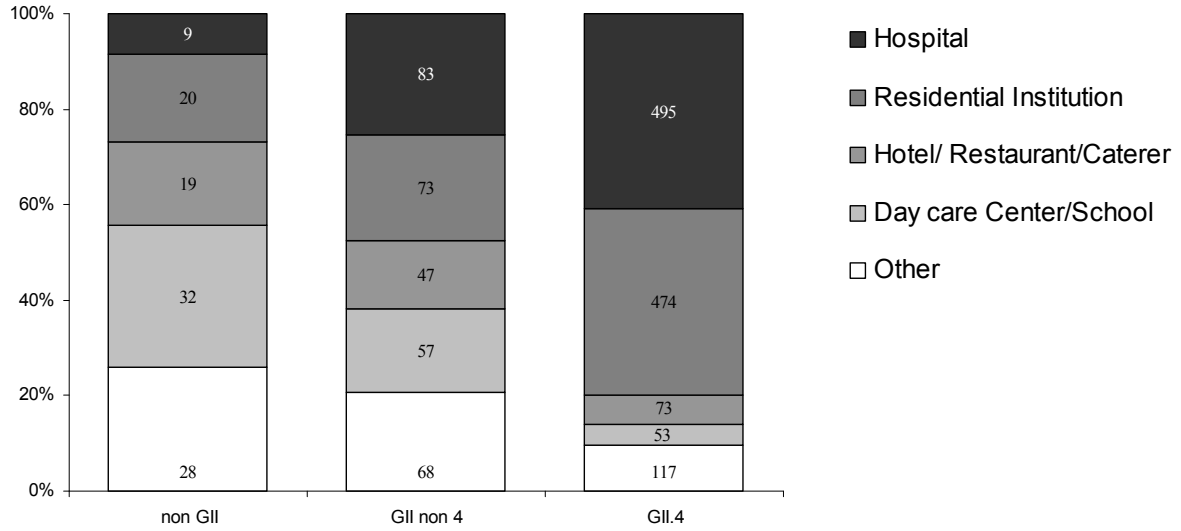

Figure 4. Setting per genotype for all genotyped outbreaks with a reported setting (n = 1,648).

Setting of Outbreaks Overall and by Genotype

Of all outbreaks with a reported setting (n = 6,579), 72% (n = 4,710) took place in a health care setting, i.e. residential institutions (36%, n = 2,383) and hospitals (35%, n = 2,327). This was similar to the proportion found in the 1,641 outbreaks for which genotyping information was available (70%, n =1,154). There was a significant difference between genotypes: 80% of GII.4 outbreaks were in health care settings, compared with 43% of the outbreaks caused by other genotypes (Figure 4). In day care centers and schools GI strains were found relatively more often. Univariate logistic regression analysis of outbreaks in health care settings showed significantly higher risk for both GII.4 (OR, 11.8; 95% CI, 7.0 to 1,086.6; P < 0.001) and other GII (OR, 2.8; 95% CI, 1.6 to 4.9; P = 0.0004) outbreaks.

Multivariate Analysis

Setting, transmission mode, and seasonality were included in a multivariate logistic regression model comparing GII.4 to other GII outbreaks. During the backward selection procedure setting and mode remained in the model as significant risk factors. No confounding from season was observed. The analysis resulted in ORs as shown in Table 2. Outbreaks in health care settings were almost nine times more likely to be caused by GII.4 strains than by NoVs belonging to genotypes other than GII. Outbreaks listed as person-to-person outbreaks were almost twice as likely to be GII.4 outbreaks.

Discussion

Our outbreak surveillance found large differences in numbers and rates of outbreaks in each country (Table 1), which are most likely a result of the differences in national surveillance systems rather than true differences in prevalence of NoV outbreaks [187, 204, 209]. This is not a specific problem of NoV surveillance, as almost all international surveillance networks cope with lack of standardization, reporting delay, and missing values in health event reporting [11, 80, 270]. In addition, NoVs are not on the list of priority diseases for surveillance in the European Union. Therefore, such differences will remain in the near future. France, Denmark, and Sweden report only suspected foodborne NoV

Table 2. Odds ratios of multinomial logistic regression, norovirus strain groups for person to person, high season and health care outbreaks*

	GII.4		GII non-4	
	OR	P value	OR	P value
Person to person†	1.9	0.026	1.0	0.8981
Health care ‡	8.8	<0.0001	2.6	0.003
N §	976		250	

*Reference category for the equation is non GG II (n=79); †suspected mode of transmission is person to person; ‡Health care = hospital or residential institution; §total N=1305.

outbreaks, and Italy and Spain do not have a national NoV surveillance system and report regional data. Norway and Ireland are new members of the network and started reporting outbreaks in 2004; Slovenia started in 2002. In Germany the system of NoV reporting to the FBVE database changed considerably during the period described in this paper. Cases of NoV infection have been notifiable since 2001 in Germany. In 2005 Germany started reporting outbreaks to the FBVE database collected through a new surveillance system. Cases are registered, and subsequently outbreaks are established by linking individual cases into groups [92]. For some of the outbreaks the virus is characterized, but due to stringent privacy laws these characterized viruses cannot be linked to individual outbreaks. Thus, although Germany has reported sequences to the FBVE database during the entire study period, these cannot be used in the overviews in this paper. Equally the structure of outbreak reporting in England and Wales, Finland, and Norway precludes systematic provision of integrated laboratory and epidemiology data. Several countries submitted additional sequences without outbreak information to the FBVE database, of which Germany and Denmark did so in large quantities (n > 200). These data cannot be used in this epidemiological overview but will be included in a phylogenetic overview elsewhere.

Taking into account the differences in the scope and system of national surveillance is necessary when doing comparative analysis of data as presented in this study [183]. In every analysis, depending on the availability and validity of the required data, a different set of outbreaks is selected.

We looked at trends in outbreak reporting, which confirmed the clear winter seasonality of NoV outbreaks [235]. The variation in the size of the seasonal peaks has been described earlier for individual countries, which reported increased activity during the 2002–2003 and 2004–2005 winter seasons [111, 168, 181, 205, 305]. However, it is difficult to draw conclusions on these trends based on surveillance data alone. Combining these data with molecular virological information, it becomes clear that the GII strains cause the seasonality and striking changes in the epidemiology have been observed: the proportion of all outbreaks caused by GII.4 rose remarkably during the observed peak years of 2002–2003 and 2004–2005 [27, 109, 305], and further analysis showed that this was in fact a succession of distinguishable variants emerging and disappearing rapidly. Molecular analysis has shown that these viruses have evolved from circulating strains by accumulation of amino acid

mutations at surface-exposed regions of the viral particle, which is highly indicative of immune-driven selection, although other explanations may exist [111, 200, 305]. The period under surveillance for the whole group is short, but data from the countries with stable surveillance over longer periods of time suggest that the high GII.4 presence and rate of change is a relatively recent phenomenon. The first observed GII.4 variant (GII.4 1996) that appeared globally was associated with high rates of outbreak reporting in 1995–1996 [356]. These viruses continued to circulate with less severe impact, until the 2002 new variant was identified through our network [205]. Again, these viruses emerged globally, and increased levels of outbreaks were reported across the world [350, 357].

New GII.4 variants have emerged every other year and disappeared a few years later, which can be seen as extremely fast evolution [41, 111, 271, 305]. This pattern is similar to observations of influenza viruses, where new variants are known antigenic drift variants, but changes in NoVs in recent years have occurred faster than those in influenza viruses [314]. Work is ongoing on global comparisons of circulating NoV variants, and preliminary results confirm that the 2004 and 2006 variants are now seen globally [308]. Although during the study period variant II.4 2006a was not found in Hungary, this variant has been reported from that country starting from October 2006. It is not yet possible to establish any direction in geographical spread, but this may be revealed in a later stage by using scan statistics [187].

The observed changes in circulating genotypes may provide part of the explanation for the apparently increased problems with NoVs in health care settings in recent years [144, 358]. Viruses of the GII.4 genotype are predominant in health care settings where people are at risk of complications of gastroenteritis [27, 28, 94, 109, 125, 206, 226]. This highly significant association, combined with the predominance of GII.4 in person-to-person transmission, suggests that GII.4 strains have properties that facilitate transmission in settings with a high concentration of people, such as higher virus loads in excreta or a higher incidence of vomiting. Quantitative data on virus shedding and on clinical symptoms are needed to test this hypothesis. A study in The Netherlands suggested increased mortality due to gastroenteritis associated with the winter seasonal peaks of 2002–2003 and 2004–2005, and similar studies are ongoing in England and Wales and France [20]. Data from the work of Verhoef et al. [341] suggested that unusual spring and summer activity can be seen as a predictor for severe winter seasons, reflecting the impact of new variant GII.4 NoVs on cruise ships as well. The unpredictable nature of this rapidly evolving RNA virus with striking changes in epidemiology is reason for concern. Understanding the mechanisms driving this process is essential to design effective intervention and prevention measures. For this a transnational approach is necessary, and thus, the need for a more standardized and systematic approach is clear, both for surveillance and for the molecular virological data collection and analysis. The global NoV initiative aims to work toward this standardized nomenclature, including recombinant strains [200, 278].

Emergence of New Norovirus Variants on Spring Cruise Ships and Prediction of Winter Epidemics

Chapter 3

Linda Verhoef, Evelyn Depoortere, Ingeborg Boxman, Erwin Duizer, Yvonne van Duynhoven, John Harris, Christina Johnsen, Annelies Kroneman, Soizick Le Guyader, Wilina Lim, Leena Maunula, Hege Meldal, Rod Ratcliff, Gábor Reuter, Eckart Schreier, Joukje Siebenga, Kirsti Vainio, Carmen Varela, Harry Vennema, and Marion Koopmans, on behalf of the Foodborne Viruses in Europe Network

Emerg Infect Dis 2008;14:238-43

Abstract

In June 2006, reported outbreaks of norovirus on cruise ships suddenly increased; 43 outbreaks occurred on 13 vessels. All outbreaks investigated manifested person-to-person transmission. Detection of a point source was impossible because of limited investigation of initial outbreaks and data sharing. The most probable explanation for these outbreaks is increased norovirus activity in the community, which coincided with the emergence of 2 new GGII.4 variant strains in Europe and the Pacific. As in 2002, a new GGII.4 variant detected in the spring and summer corresponded with high norovirus activity in the subsequent winter. Because outbreaks on cruise ships are likely to occur when new variants circulate, an active reporting system could function as an early warning system. Internationally accepted guidelines are needed for reporting, investigating, and controlling norovirus illness on cruise ships in Europe.

Introduction

Norovirus is a highly infectious causal agent of a usually mild and self-limiting acute gastroenteritis. The symptoms of vomiting and diarrhea occur after a short incubation period of 8 to 72 hours. Although norovirus can cause sporadic cases [73], this contagious virus is often described as a cause of outbreaks [41, 93, 271, 294]. In Europe, norovirus outbreaks are reported to the Foodborne Viruses in Europe (FBVE) network. This network maintains a Web-based surveillance database containing data reported by 13 European countries [174].

In June 2006, the Dutch Food and Consumer Product Safety Authority (VWA) contacted the coordinator of the FBVE network, located at the National Institute for Public Health and the Environment (RIVM) in the Netherlands. The VWA had been notified of suspected norovirus outbreaks on 3 cruise ships operating in the Netherlands in the previous month. In the same week, ProMED reported a viral gastroenteritis outbreak on a Dutch-owned cruise ship operating out of the United Kingdom [277]. Norovirus outbreaks on cruise ships are not normally reported to national surveillance centers in Europe, but having been alerted to these outbreaks, these centers recognized that the number of outbreaks was unusual. In Europe, norovirus outbreaks are highly seasonal, with most outbreaks reported from October through April [207, 208]. Further inquiries found that passengers on several ships sailing within European waters were experiencing outbreaks of gastroenteritis. This finding resulted in a coordinated investigation between the European Centre for Disease Prevention and Control (ECDC) and the FBVE network to identify or exclude a common source of infection [176, 324]. The investigation was based on the hypotheses that the possible rise in reported outbreaks was 1) reporting bias resulting from media attention and active investigation of these outbreaks, 2) an actual increase specific for cruise ships by means of a common source, or 3) a reflection of actual increased norovirus activity in the community. We describe the results of data collection at the European level by an international and multidisciplinary investigation team.

Methods

Epidemiologic, virologic, and baseline data were collected from various sources. These sources included the FBVE network, ECDC, Food Safety Authorities, Early Warning

Response System messages, diagnostic and reference laboratories, local health institutions, and ship owners.

Definitions

A single outbreak was defined as a cluster of at least 3 people becoming ill within 3 days of each other with symptoms of acute gastroenteritis during 1 voyage with 1 group of passengers on board a ship. A ship-level outbreak was defined as successive single outbreaks occurring on 1 ship. An outbreak was confirmed if norovirus was detected in stool samples from >2 patients and was considered probable if norovirus was detected in only 1 patient's sample or in >1 environmental samples. If descriptive clinical data suggested a viral cause but microbiologic proof for the causative agent was absent, the outbreak was considered as possibly caused by norovirus. Because norovirus outbreaks typically occur in winter, we defined a norovirus surveillance year as running from May through April of the next year to include a full winter season. Two periods were defined: off-seasonal, lasting from May through September; and seasonal, lasting from October through April of the following year.

Data Collection

Epidemiologic Data: We included outbreaks that occurred on ships sailing within Europe and that were reported between January 1 and August 1, 2006. Information describing the outbreaks was collected; the dataset is given in a footnote of Table 1. If an on-site outbreak investigation was performed, local authorities were asked to send their outbreak report to the investigation team.

Virologic Data: Environmental samples and patients' stool samples were collected and were tested at different institutions by using local protocols, primarily reverse transcription–PCR [348]. Virus information was collected to determine the causative agent and to determine whether identical strains indicated a common source for different ships. Sequences from a specific genomic region, the polymerase region A, were analyzed, which allowed an international comparison to be made [338]. If this analysis could not be done at a local level, stool samples, RNA, or sequence information was sent to reference laboratories.

Background Data: The FBVE database [174] enables analyses of combined epidemiologic and virologic data. Baseline incidence was determined by analyzing the reported outbreaks, as registered in the FBVE database in March 2007. Outbreaks with onset from May 2002 through February 2007 were selected for the analyses. Because data from surveillance data collection were incomplete and associated with delays, to assess the number of outbreaks that occurred from May through June 2006, the FBVE network conducted an email survey within the network in July 2006. In addition, outbreak data for 2006 were obtained from Australia and Hong Kong and compared with data for 2005.

Data Analysis

Epidemiologic Data: The following data were obtained retrospectively from outbreak reports: number and duration of outbreaks, overall attack rate, attack rates among passengers and crew, availability of (adequate) protocols and materials for cleaning, passenger flow during embarkation and disembarkation (possibility of contact between arriving and departing passengers), sick leave for crew, and policy for sick patients (isolation or not). For comparison of proportions, p values were calculated according to the χ^2 test and Fisher exact test, if appropriate. Available epidemic curves were used to determine whether

a point source infection was indicated through log-normal distribution [291], with patients clustering within 1.5× the incubation period range (CDC Manual, available from www.cdc.gov/health/botulism.htm; the manual was adapted for general epicurves, available from www.epi.state.nc.us/epi/gcdc/manual/Epicurves.pdf). Data were analyzed by using PEPI 4.0 (Programs for Epidemiologists; Sagebrush Press, Salt Lake City, UT, USA).

Virologic Data: Nucleotide sequences were aligned through Bionumerics software version 4.6 (Bionumerics package, Applied Maths, Ghent, Belgium). These sequences were then compared with consensus sequences by using a publicly available, web-based, quick-typing tool (available from www.rivm.nl/bnwww).

Background Data: The differences between the off-seasonal number of reported outbreaks in 2006 (x_1) and the off-seasonal numbers of reported outbreaks in previous years ($x_{2–5}$) were compared; the difference was significant according to the following equation:

$$\frac{N(x_1 - N(x_2)}{\sqrt{Nx_1 + Nx_2}} > 1.96$$

In addition, we compared the numbers of off-seasonal, seasonal, and cruise ship outbreaks in 2006 and 2007 with the numbers from previous years, from the FBVE database. Annual seasonal numbers of reported outbreaks were ranked to assess Spearman rank correlation coefficients. Poisson regression analysis was performed to determine whether the numbers of off-seasonal outbreaks were independent from the numbers of cruise-related outbreaks. In the FBVE database, outbreaks that occur on ships are reported in the category aircraft/ship/train/bus; analysis of cruise ship outbreaks used this category, which led to some misclassification of cruise ship outbreaks. Some additional information on setting is given in free text fields and, if available, was used to reduce misclassification. Data were analyzed by using SAS 9.1 for Windows (SAS Institute Inc., Cary, NC, USA).

Results

During the study period, 43 single outbreaks were reported from 13 vessels: 14 (33%) of these were confirmed, 2 (5%) were considered probable, and 27 (63%) were considered possible norovirus outbreaks. For ship-level outbreaks, norovirus infection was confirmed for 10 (77%) vessels, 1 (8%) ship had probable norovirus infections, and 2 ships (15%) had possible norovirus infections.

Epidemiologic Data

Of the 43 outbreaks, 1 occurred in January 2006; all others occurred from April 24 through July 21, with only a 2-week outbreak-free period (Figure 1). Three outbreaks on 3 ships occurred during the season; 40 outbreaks on 10 ships occurred during off-season months. Table 1 shows available epidemiologic and virologic data for each cruise ship that had confirmed or probable norovirus outbreaks. Overall attack rates varied from <1% to 41%. The highest attack rates were 48% for passengers and 19% for crew members. Ships 10 and 13, which were ferries, reported the lowest overall attack rates and higher attack rates for crew than for passengers ($p = 0.021$ for ship 10; $p = 0.064$ for ship 13).

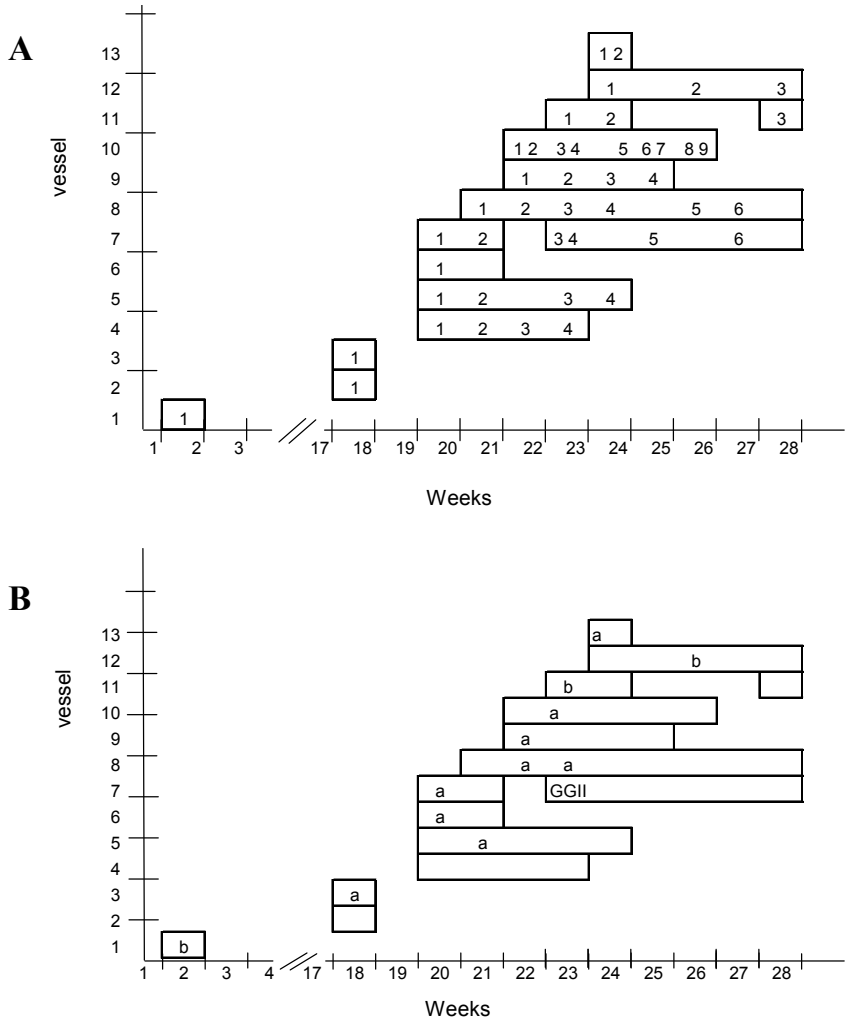

Figure 1. Number of outbreaks (A) and causative genotypes (B) for cruise-related outbreaks of norovirus for each ship from January through July 2006. Data were derived from multiple sources, active case finding, and case reports. a, GGII.4–2006a; b, GGII.4–2006b; GGII, GGII but variant unknown.

Source of the Outbreaks

For 3 ships, the epidemic curve of the initial outbreak was available. One of these indicated a point source, which could not be identified during the outbreak investigation. For 7 of the 13 ships, at least 15 food suppliers were identified. Of these, 13 suppliers delivered products to 1 ship and 2 suppliers delivered to multiple ships (Table 1). A common food source could not be identified for all ships. For 6 ships, no information on the food supplier was available.

A retrospective cohort study performed on 1 ship and case-control studies on 2 other ships could not find any evidence of a point source. Person-to-person spread was believed to be the predominant route of transmission as shown in these analytical studies and in another 6 descriptive reports.

Risk Factors for Multiple Outbreaks

Reports from local investigation teams were available for 7 of 9 ships that experienced multiple outbreaks and for 2 of 4 ships that experienced only 1 outbreak. These

Table 1. Characteristics of outbreaks of gastroenteritis on vessels with at least one confirmed or probable norovirus outbreak, sailing within Europe and reported between January 1st and August 1st, 2006*

Ship	Owner	Food supply	Point source first OB	OB no.	Duration in days	OB definition	Completeness (%)††	On-site investigation	Attack rate total		Attack rate passengers		Patient samples Pos (taken)	Sequence patient sample	Environ. samples pos (taken)	Sequence environmental sample
									At risk	ill (%)	At risk	ill (%)				
1†	*A*	-	-		-			*No*	-	-	-	-		*GGII.4-2006b*	-	-
				1	-	Confirmed	36	No	-	-	-	-	3 (5)	GGII.4-2006b	-	-
3†	*B*	-	-		-			*Yes*	*140*	*15 (11)*	-	-	*0 (1)*	-	*2 (4)*	*GGII.4-2006a*
				1	-	Probable	79	Yes	140	15 (11)	-	-	0 (1)	-	2 (4)	GGII.4-2006a
5†	*E*	*X, others*	*No*		*30*			*Yes*	*2590*	*104 (4)*	-	-	*6 (7)*	*GGII.4-2006a*	-	-
				2	9	Confirmed	82	Yes	659	69 (10)	465	69 (5)	4 (4)	GGII.4-2006a	-	-
				4	2	Confirmed	75	Yes	636	3 (<1)	450	3 (<1)	2 (3)	-	-	-
6†	*D*	*Y*	*No*		-			*Yes*	*166*	*61 (37)*	-	-	*4 (5)*	*GGII.4-2006a*‡	*3 (3)*	*GGII.4-2006a*‡
				1	-	Confirmed	96	Yes	166	61 (37)	132	57(43)	4 (5)	GGII.4-2006a	3 (3)	GGII.4-2006a
7†	*F*	-	-		*>22*			*Yes*	*4042*	*342 (8)*	-	-	*≥2 (17)*	*GGII.4-2006a*	*0 (0)*	-
				1	7	Probable	79	Yes	725	108 (15)	504	90 (18)	≥1 (2)	GGII.4-2006a§	-	-
				4	-	Confirmed	57	No	700	39 (6)	500	38 (8)	≥1 (10)	GGII	-	-
8†	*C*	*X, others*	-		*>29*			*Yes*	*3578*	*293 (8)*	-	-	*15 (17)*	*GGII.4-2006a*	*0 (0)*	-
				2	6	Confirmed	68	Yes	575	7 (1)	-	-	6 (6)	GGII.4-2006a¶	-	-
				3	6	Confirmed	79	Yes	593	120 (20)	402	117 (29)	6 (6)	GGII.4-2006a¶	-	-
				6	-	Confirmed	57	Yes	600	5 (<1)	-	-	3 (5)	-	-	-

Ship	Owner	Food supply	Point source first OB	OB no.	Duration in days	OB definition	Completeness (%)[††]	On-site investigation	Attack rate total		Attack rate passengers		Patient samples pos (taken)	Sequence patient sample	Environ. samples pos (taken)	Sequence environmental sample
									At risk	ill (%)	At risk	ill (%)				
9[†]	*G*	*-*	*-*		*-*			*Yes*	*12124*	*369 (3)*	*-*	*-*	*8 (11)*	*GGII.4-2006a*	*1 (≥1)*	*-*
				1	-	Confirmed	75	Yes	3124	234 (7)	2261	220(10)	8 (11)	GGII.4-2006a	1 (≥1)	-
10[†**]	*H*	*Z*	*-*		*>3*			*Yes*	*10013*	*40 (<1)*	*-*	*-*	*3 (3)*	*GGII.4-2006a*	*0 (3)*	*-*
				4	1	Confirmed	71	Yes	1139	6 (<1)	1011	3 (<1)	3 (3)	*GGII.4-2006a*[§]	-	-
11[†]	*I*	*-*	*-*		*-*			*No*	*2217*	*68 (3)*	*-*	*-*	*8 (8)*	*GGII.4-2006b*	*0 (0)*	*-*
				1	-	Confirmed	54	No	350	34 (10)	-	-	8 (8)	GGII.4-2006b	-	-
12[†]	*D*	*Y*	*-*		*>30*			*Yes*	*454*	*137 (30)*	*-*	*-*	*6 (7)*	*GGII.4-2006b*[‡]	*4 (13)*	*GGII.4-2006b*[‡]
				2	9	Confirmed	100	Yes	147	61 (41)	115	55 (48)	6 (7)	GGII.4-2006b	4 (10)	GGII.4-2006b
13[†**]	*J*	*X, others*	*-*		*8*			*Yes*	*2442*	*40 (2)*	*2142*	*32 (1)*	*7 (10)*	*GGII.4-2006a*[‡]	*1 (10)*	*GGII.4-2006a*[‡]
				1	1	Confirmed	100	Yes	1150	33 (3)	1000	25(3)	4 (5)	GGII.4-2006a[¶]	0 (5)	
				2	3	Confirmed	89	Yes	1292	7 (<1)	1142	7(<1)	3 (5)	-	1 (5)	GGII.4-2006a[¶]
Total	**10**								**37766**	**1469 (4)**			**≥58 (87)**		**11 (≥31)**	

*For ships 2 and 4 none of the outbreaks could be contributed to norovirus; these ships are excluded from this table; [†] Single outbreak= a cluster of at least 3 people becoming ill within 3 days of each other with symptoms of acute gastroenteritis during one voyage with a single group of passengers on board a ship. Ship-level outbreak= successive single outbreaks occurring on one ship (numbers are presented in italic); [‡] Identical sequences detected in environmental and patient samples; [§] Several mutations compared to GGII.4-2006a in other outbreaks and identical for ships no. 7 and 10; [¶] One mutation compared to GGII.4-2006a in other outbreaks and unique when compared to strains from other vessels; [#] If exact numbers of cases or population at risk were missing, estimates obtained from the ship owners were used.

low numbers, including missing values, did not enable analysis to identify risk factors for multiple outbreaks. Descriptive information indicated the following risk factors: possible contact between boarding and disembarking passenger groups and cleaning with inappropriate materials for norovirus elimination during the first outbreak.

Virologic Results

The norovirus sequences, detected in fecal or environmental samples, were all of the GGII.4 genotype but in 2 distinct new lineages, designated GGII.4–2006a and GGII.4–2006b (Table 1 and Figure 1) [306]. Samples taken from 8 (73%) and 3 (27%) of 11 ships were identified with the GGII.4–2006a and GGII.4–2006b variant, respectively.

For 3 ships, the norovirus strains obtained from environmental samples were genetically identical to those obtained from patient samples. Positive environmental samples were derived from contact surfaces, which implied that person-to-person transmission through aerosols and contact with contaminated surfaces was possible. For 1 ship, samples of raspberries and tap water taken during the outbreak were found to be contaminated with norovirus. Whether the contamination was the source of the outbreak or resulted from contact with patients affected in the outbreak could not be determined.

Analysis of Background Norovirus Activity

FBVE Database: From May 1, 2002, through February 28, 2007, a total of 9,425 norovirus outbreaks were reported to the FBVE network. A total of 2,480 outbreaks occurred during the norovirus surveillance year 2006–2007. For 8 of the countries, analysis of the number of off-season outbreaks from 2002 through 2006 was possible. A combined total of 137 outbreaks were reported by these countries during the 2006 off-season. This number is higher than that for the same months in 2003 (n = 68, significant), 2004 (n = 127, not significant) and 2005 (n = 132, not significant) but lower than that for 2002 (n = 383, significant) when norovirus activity was very high. However, reporting for the year 2004–2005 has been considerably delayed (median 157 days, range 4–616). Since data were derived from the database on March 14, 2007, the numbers of reported outbreaks in the surveillance database are still increasing. An average of 5 aircraft/ship/train/bus-related outbreaks per year is reported in the FBVE database (Figure 2). The Spearman rank correlation coefficient was significant between the number of outbreaks in this category and the number of off-season outbreaks ($R = 0.97$; $p = 0.0048$). Poisson regression analysis showed that the annual number of off-season cruise ship outbreaks associated strongly with the total annual numbers of outbreaks in the subsequent season ($p = 0.0078$) as well as the same off-season ($p<0.001$). In the norovirus surveillance year 2005–2006, GGII.4 strains were the predominant type identified (79%). The 2 new lineages within the GGII.4 genotype were first detected between January and March and displaced the resident GGII.4–2004 strains (Figure 2).

Survey

Of 13 collaborating countries in the FBVE network, 11 reported higher norovirus activity at the time of the ongoing cruise ship outbreaks. Australia and Hong Kong [267] experienced higher norovirus activity from January through June 2006,

compared to the same period in 2005: a more than 10-fold increase was reported in Adelaide (147 for 2006, 117 of which occurred out of season from January through July), and Hong Kong reported 99 outbreaks from January through June 2006 compared with 46 outbreaks in 2005. In Australia and Hong Kong, outbreaks were associated with the new lineages 2006a and 2006b, respectively.

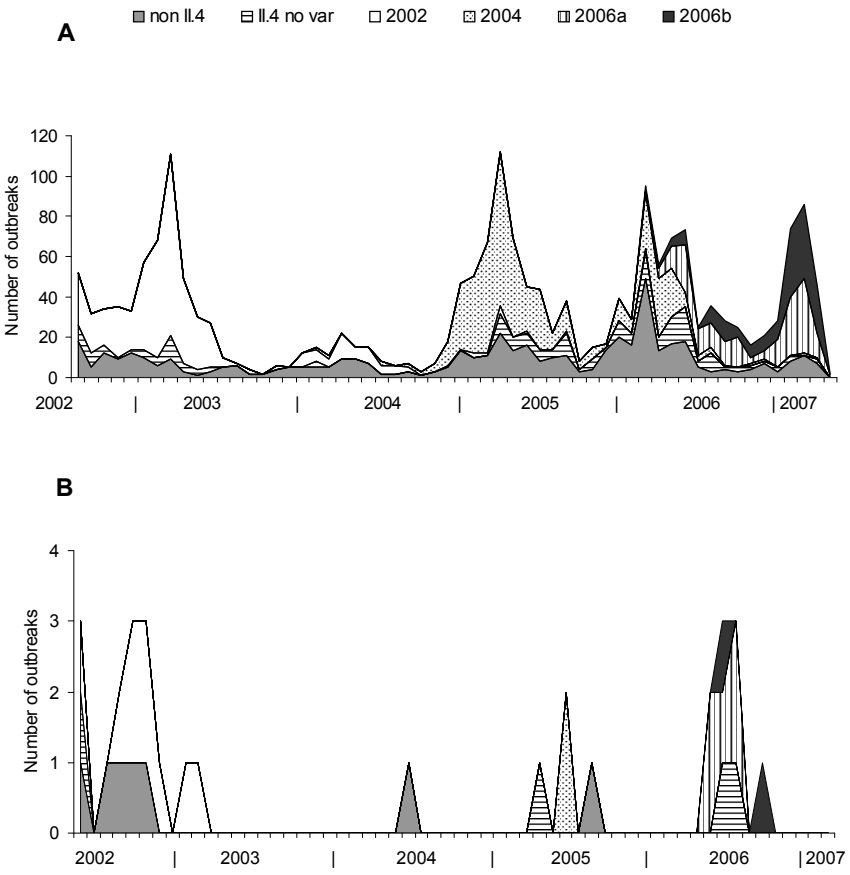

Figure 2. Cumulative outbreak data over time (2002–2007) from Foodborne Viruses in Europe network database. The total number of reported outbreaks (A) contrasted with the reported ship-related outbreaks (B). Both show norovirus strains involved.

Discussion

An unexpectedly high number of outbreaks on cruise ships in European waters in the spring and summer of 2006 triggered this investigation. Concomitant with this increase of norovirus outbreaks on cruise ships, we noted a marked increase in norovirus activity in the general population. The overall increase in norovirus activity in summer 2006 coincided with the emergence of 2 new norovirus GGII.4 strains and was followed by a higher number of outbreaks than usual in winter in 2006–2007 [182]. Reporting of cruise ship related norovirus outbreaks may have been influenced by heightened attention from the media; however, the increase in reported land-based outbreaks is indicative of a real increase.

A similar situation occurred in the spring and summer of 2002, when a new variant of the GGII.4 strain emerged globally. This variant was found on cruise ships through the US Vessel Sanitation Program and in nursing homes and hospitals through the FBVE network [47, 205, 357]. Retrospective analysis of 5 years of surveillance data from the FBVE network also showed a correlation between the number of off-season outbreaks on cruise ships and higher norovirus activity in the subsequent winter season. This recurring situation implies that cruises are possibly an early indicator for increased norovirus activity in the community because they are highly susceptible to norovirus outbreaks and mostly sail during warmer months of the year. A prospective and active surveillance program could demonstrate the validity of cruise ship outbreak incidence as a predictor of norovirus activity for the next season. After the first infection is introduced in this closed setting, an outbreak is likely to occur through person-to-person transmission [207]. With the regular changing of passenger groups, the noroviruses on board are able to repeatedly infect a new susceptible population [257]. Exhaustive control measures may not always be sufficient to eliminate the virus; a striking example is a positive environmental swab from a handle of a hand sanitation container, which was used before entering a restaurant. To get a better understanding of the epidemiology of noroviruses aboard cruise ships, we need a definition for a single outbreak that is more stringent than the one we used. That will only be possible with some level of routine monitoring of illnesses.

Our results could neither indicate nor exclude a point source or a common link through food or water. Information on food supply was incomplete and difficult to obtain. Separation of a potential point source from person to person or environmental transmission can optimally be investigated during a ship's initial outbreak. This investigation was only conducted on 1 ship. That 2 different lineages of GGII.4 norovirus were involved provided some evidence that a common source for all ships was unlikely (Figure 1). That a common source was unlikely was further supported by the background data showing the emergence of the same viruses coinciding with increased reporting of outbreaks from all kinds of settings across the network (Figure 2).

Attack rates for crew and passengers differed. Attack rates for the crew were mostly lower than rates for passengers, which may have been due to short-term immunity, possibly acquired during successive outbreaks over long periods [149, 199]. However, reporting bias is possible because crew members may be reluctant to admit to being ill [101]. The only 2 ships in which attack rates were higher for the crew were the 2 ferries. This finding is likely due to an underestimation of number of ill passengers, because their stay on board is shorter than the average incubation period. This explanation is supported by the fact that some ferry passengers were coincidentally discovered to have been ill during their return trip 2 days later.

Patient samples are needed to confirm the causative agent of the gastroenteritis outbreaks and to analyze the genetic sequence of viruses. Typing the norovirus strain will help show whether the outbreak is likely the result of reintroduction of the virus through a person. Person-to-person transmission is likely when community norovirus prevalence is high [169] and is a situation that shipping companies may be unable to prevent even if they are adhering to good cleaning

procedures. Unfortunately, reintroduction of the virus through a new strain could not be determined in our study for 2 reasons. First, patient sampling when passengers fall ill on cruise ships in Europe is not standardized. Virus genotyping data for subsequent outbreaks were acquired from only 1 ship, where they were identical. Second, our data may have been insufficient to discriminate between GGII.4 variants and to determine sequence diversity. New variant strains of GGII.4 emerged over a vast geographic region within a short period of time, resulting in the finding of similar or identical sequences in outbreak strains collected throughout Europe. The level of genome analysis needed to enable discrimination between individual outbreak strains remains to be determined [212].

International outbreak surveillance can 1) provide background data on baseline activity of the virus and circulating strains and 2) facilitate tracing of foodborne sources, especially in the case of diffuse outbreaks that may result from centralized production and wide geographic distribution of products. At times of unusual numbers of outbreaks, additional active data collection helps compensate for underreporting, reporting delays, and helps elucidate export routes of foods. In the situation described here, thorough outbreak investigation was complicated as a result of continuation of trips through different countries during the course of outbreaks. This problem is a point of concern during potential common-source outbreaks, in which early detection of the source is crucial; this matter was considered by the ECDC, which launched an initiative for measures in Europe [76].

Gastroenteritis outbreaks on cruise ships may seem a luxury problem. However, at times of increased norovirus activity they are likely to occur and to receive much media attention. Higher norovirus activity appeared to coincide with emergence of new variant GGII.4 strains and with a higher number of cruise-related outbreaks in the preceding spring and summer. Cruise ship holidays create an environment in which norovirus is easily spread and outbreaks readily occur. Therefore, a reporting system for cruise ship–related outbreaks of gastroenteritis including virus detection and typing may function as an early warning system for high-epidemic winters. Such a system may enable a quick response and minimize negative effects of increased norovirus activity.

Multiple Exposures during a Norovirus Outbreak on a River-cruise Sailing through Europe, 2006

Chapter 4

Linda Verhoef, Ingeborg L. Boxman, Erwin Duizer, Saskia A. Rutjes, Harry Vennema, Ingrid H. M. Friesema, Ana-Maria de Roda Husman, Marion Koopmans

Euro Surveill 2008;13:pii=18899

Abstract

In the summer of 2006, several cruise-related viral gastroenteritis outbreaks were reported in Europe. One report came from a river-cruise, belonging to a ship-owner who had two other ships with outbreaks. This situation warranted onsite investigation in order to identify a potential common source of infection. A retrospective cohort study was performed among 137 people on board. Epidemiological questionnaire data were analyzed using logistic regression. Stool, food, water and surface samples were collected for norovirus detection. Norovirus GGII.4-2006b was responsible for 48 gastroenteritis cases on this ship as confirmed in six patients. Identical norovirus sequences were detected in stool samples, on surfaces and in tap water. Epidemiological and microbiological data indicated multiple exposures contributing to the outbreak. Microbiological results demonstrated person-to-person transmission to be clearly present. Epidemiological results indicated that consuming tap water was a risk factor; however, this could not be concluded definitively on the basis of the available data. A common source for all cruise-related outbreaks was unlikely. The ongoing outbreaks on this ship demonstrated that evidence based guidelines on effective disinfection strategies are needed.

Introduction

Noroviruses are a well known viral cause of acute gastroenteritis (GE) on cruise ships [13, 357]. Most norovirus outbreaks on cruise ships are described as being caused by person-to-person transmission. The virus is persistent and eradication is complicated in such closed settings [146]. On 3 July 2006, the National Institute for Public Health and the Environment (RIVM) in the Netherlands was notified of an outbreak of GE with characteristics indicating a viral agent. The outbreak occurred during successive voyages of a river-cruise ship sailing through several European countries. The outbreak was one of a large cluster of cruise-ship-related outbreaks reported in Europe at that time [176, 324, 341], all of which had ascribed norovirus as the causative agent. Moreover, it was the third ship from one company reporting ships with outbreaks and one of four ships that was dealing with GE while sailing through the Netherlands. In addition, this notified outbreak had endured consecutive voyages from 11 June 2006, despite sanitation measures, triggering questions about a possible and persisting common cruise-ship-related source of infection other than person-to-person transmission. An investigation by an outbreak investigation team was initiated to identify a possible source of infection. The ship docked in Nijmegen in the Netherlands on 6 July 2006, and provided the opportunity to undertake an onsite investigation.

Methods

A retrospective cohort study was performed among passengers and crew joining the second of three successive voyages, being further referred to as the current voyage, of this ship affected by outbreaks of GE. The outbreak investigation

team included two epidemiologists from the National Institute for Public Health and the Environment (RIVM) and an inspector from the Food and Consumer Product Safety Authority (VWA). This team interviewed the ship's captain and hotel manager, following a structured questionnaire that focused on the origin of viral GE outbreaks. Information concerning cleaning procedures was collected. Passengers and crew, joining the ship's current voyage affected by GE, were provided with a questionnaire that they completed individually. Stool, food and drinking water samples together with surface swabs were collected.

Epidemiological Data Collection

The starting date, the menu cycle and information of facilities on board during the current voyage were used to prepare a questionnaire. The questionnaire is available from the authors by request. All food items from the menu served between boarding time on 25 June 2006 and breakfast on 27 June 2006 were included, considering an incubation period of 12-72 hours of the first reported symptomatic person. The following additional risk factors were addressed: water use, public toilet use and contact with infected people. To assess the potential introduction of the virus by a person, history of GE during the week preceding the cruise trip was asked. To allow the grouping of respondents with respect to biological plausible risk from food consumption according to the incubation period, the exact starting and ending time of symptoms were collected.

To determine the potential initial introduction of the causative agent through a person, the person who was the first to report symptoms was contacted. This occurred during the previous and first voyage of this ship affected by GE. This person – the index case – was interviewed by telephone using a questionnaire adjusted to the menu during his voyage.

Virological Data Collection

Stool Samples: Twenty packages for the collection of stool samples, together with a detailed instruction form, were left on board the ship with the people responsible for passenger health. Stool samples were sent to the RIVM by overnight mail and then stored at 4˚C. This is a commonly accepted procedure for the stable norovirus; furthermore, it increases the response rate for stool sample collection [150]. Crew members were instructed to approach at least five symptomatic and five asymptomatic people for stool sampling. Stool samples were tested for the presence of norovirus, as this virus was the suspected causative agent causing cruise-related outbreaks of GE in Europe at that time [176, 324, 341]. For confirmation of a norovirus outbreak, at least two of five case-originating samples need to be tested positive [87]. Stool samples were analyzed as described by Svraka et al. [323]. Genotyping was done by sequence analysis of a fragment of the ribonucleic acid (RNA) dependent RNA polymerase gene, as described previously [348].

Potential Source Samples: The VWA collected food, water and surface samples according to a protocol designed to avoid cross-contamination of samples. Before sampling tap water, taps were cleaned with alcohol and contact between tap and sample bottle was avoided. Environmental swabs were analyzed using a method which is described elsewhere [34]. Food samples were analyzed for the presence of norovirus according to in-house protocols using (nested) real time polymerase chain

reaction (PCR) assays [32, 33]. One of the food samples – raspberries – was also analyzed in three other specialized food laboratories in France, Finland and the Netherlands, according to local protocols. Water samples of one liter were filtered through a positively charged membrane and detected according to Van den Berg et al. [334].

Data Analyses

As in most European countries, in the Netherlands microbiological diagnosis of norovirus is outbreak-based [87] instead of case-based. To identify norovirus patients, we used the following definition for acute GE: at least two episodes of diarrhea and/or at least two times of vomiting within 24 hours. Discrimination was made between early cases and late cases, to determine biological plausible exposure from food addressed in the questionnaire. Early or late cases were characterized as people in whom illness occurred within or after, respectively, 72 hours after the last breakfast included in the questionnaire, i.e. before or after, respectively, 30 June 2006 10 a.m. To determine biological plausible risk from specific food items, the exact onset of disease was compared to the serving moment of the food item while assuming an incubation period of 12 to 72 hours.

Relative risk was calculated for all questionnaire items. Significant and biological plausible risk factors were analyzed using a multiple logistic regression model to determine relevant factors after adjustment for potential confounders. Proportions were compared calculating p-values according to X^2 if numbers were sufficient; Fisher's exact test was applied if cells in cross tables contained five or fewer records. All results are presented including 95% confidence intervals (95% CI). Data were analyzed using SAS 9.1 for Windows (SAS Institute Inc., Cary, NC, USA).

Results

The voyage started on 25 June 2006, and ended on 9 July 2006, while docking in 12 cities in Switzerland, France, Germany, the Netherlands and Belgium (Figure 1).

Epidemiological Results

During the outbreak 33 crew members from Romania (n=8), Slovakia (n=7), Hungary (n=3), Bulgaria (n=3), Croatia (n=3), the Netherlands (n=3), Poland (n=2), Serbia (n=2) and Germany (n=2), and 104 passengers from the United States of America (n=102) and the United Kingdom (n=2) were on board. Of these 137 people at risk, 48 (35%) met the case definition of acute GE. Questionnaires from 29 (88%) crew members and 98 (94%) passengers were returned (Table 1). Of these, 2 (7%) crew and 46 (47%) passengers met our case definition for acute GE, with crew having a significantly lower attack rate ($p<0.001$). The epidemic curve for the cases showed a clear peak in the number of reported cases on day 4. The somewhat tailed distribution suggested a secondary wave of cases (Figure 1). Two Romanian crew members who had recently entered the ship during the current voyage reported symptoms of GE the previous week when at home.

Table 1. Characteristics and case-definitions of the population at risk during an outbreak of gastroenteritis during a river-cruise

Characteristic	Crew (n=31) *	Passengers (n=98) *	Total (n=129)*
Mean age (range)	29.4 (20-43)	69.3 (14-87)	59.7 (14-87)
Sex male / female	19 / 12	47 / 50	66/62
Symptomatic / asymptomatic persons based on			
Case definition for acute gastroenteritis (AGE) †	2 / 27	46 / 45	48 / 72
Case definition AGE and plausibility food risk‡	1 / 27	10 / 45	11 / 72

* Questionnaire-data of 2 crew members and 6 passengers are missing.

† At least two episodes of diarrhea and/or at least 2 times of vomiting within 24 hours.

‡ Food-items addressed in the questionnaire a time-span from boarding time through breakfast at 27 June, 2006 If the requested food item was consumed within 12-72 hours before onset of illness, risk from this food item was considered biological plausible.

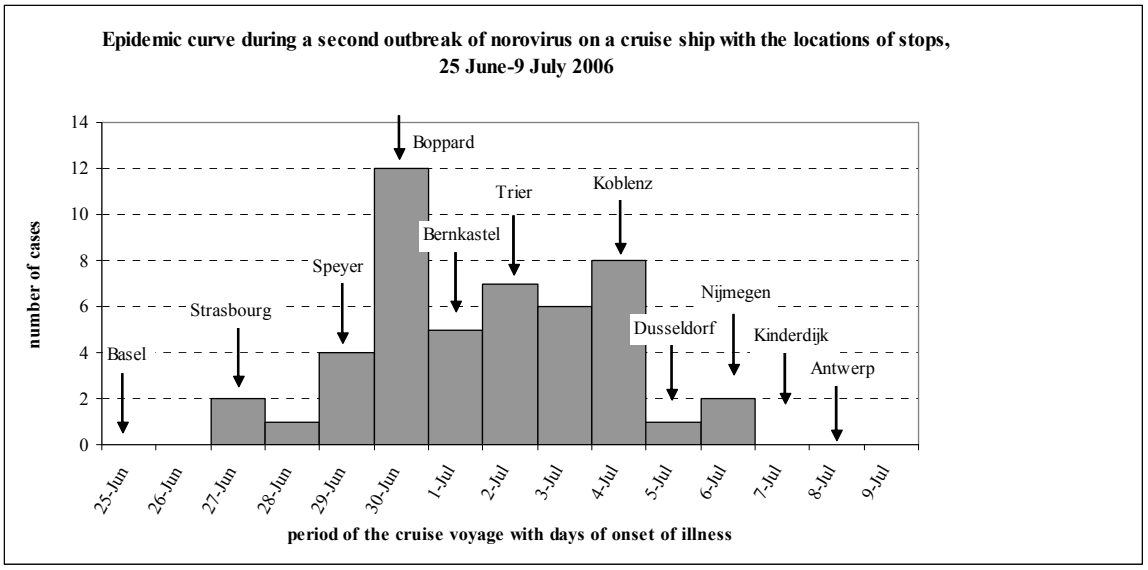

Figure 1. Epidemic curve during a second outbreak of norovirus on a cruise ship with the locations of stops, 25 June – 9 July 2006.

Over 100 food items and five behavioral risk factors were addressed in the questionnaire. Of these, possible risk factors for cases in univariate analyses were: contact with a sick room-mate, and consumption of egg, carrot pie, tap water and whipped cream (Table 2). The number of early cases was too low for univariate analysis; however, results indicated tap water, ice cubes, egg consumption and a sick room-mate as possible risk factors. When restricting the plausible exposure from these factors to illness within 72 hours after consumption, the risk from egg, carrot pie and whipped cream consumption was considered unlikely: only eight of 38 ill egg consumers, three of 15 ill carrot pie consumers and two of nine ill whipped cream consumers became ill within 72 hours of consumption of the food item. Raspberries were not a significant risk factor when served as 'raspberry yoghurt'. Raspberries

were also used as garnish, but this was not mentioned on the menu and thus not requested in the menu-based questionnaire. Use of public toilets on board could not be investigated as a risk factor, since public toilets had been closed a few hours after boarding time. This measure was introduced based on the outbreak during the ship's preceding voyage.

Table 2. Relative risk (95%CI) and biological plausibility for having acquired a norovirus infection on board of a river-cruise ship within the time-span addressed in the questionnaire

Risk factor	All cases RR(95%CI)	Early case* RR(95%CI)	Late case† RR(95%CI)	Plausibility‡
Tap water	**2.8 (1.4-5.6)** §	2.6 (0.7-7.3)	**2.8 (1.3-6.0)**	**Yes**
Ice cube use	1.7 (0.9-3.1)	3.8 (0.6-25.4)	1.4 (0.7-2.6)	Yes
Fresh juice	1.4 (0.9-2.1)	2.0 (0.7-5.3)	1.1 (0.8-1.8)	Yes
Sick room-mate	**2.2 (1.3-3.6)**	3.3 (0.9-11.7)	**1.9 (1.1-3.1)**	**Yes**
Egg	**2.9 (1.5-5.8)**	4.6 (0.7-29.5)	**2.5 (1.3-5.2)**	**No**
Carrot pie	**1.3 (1.0-1.6)**	1.2 (0.8-1.7)	1.3 (1.0-1.7)	No
Whipped cream	**1.2 (1.0-1.4)**	1.2 (0.9-1.5)	1.2 (1.0-1.4)	No

* Early cases were characterized as persons in whom illness occurred within 72 hours after the last breakfast included in the questionnaire, i.e. before 30 June 2006 10 a.m.

† Late cases were characterized as persons in whom illness occurred after 72 hours after the last breakfast included in the questionnaire, i.e. after 30 June 2006 10 a.m.

‡ Food-items addressed in the questionnaire a time-span from boarding time through breakfast at 27 June, 2006 If the requested food item was consumed within 12-72 hours before onset of illness, risk from this food item was considered biological plausible.

§ Significant risks are presented in bold (n=120).

For juice and ice cubes, tap water was needed for preparation. Therefore, juice-drinkers and ice cube users were added to the water consumers in order to account for misclassification. Relative risk from water consumption became 3.2 (1.3-7.8) when combined with ice cube users and 4.2 (1.3-13.5) when combined with juice drinkers, suggesting a potential dose-response relationship. Adding both ice cube users and juice drinkers to the water consumers resulted in a relative risk of 3.3 (0.9-12.3). In a multiple logistic regression model, the consumption of tap water, ice cubes, orange juice and whipped cream were corrected for person-to-person transmission through the variable 'sick room-mate'. In this model, water - either with or without accounting for misclassification - remained a statistically significant risk.

In the outbreak during the previous voyage, 47 of 147 (32%) people on board presented with symptoms of gastroenteritis. These illness reports were poorly recorded with consequent missing dates of onset, and no attack rates specified for passengers and crew. Reportedly, none of the patients had vomited in a public area on board. A telephone interview with the index case took place at 27 July 2006, which was 6 weeks after the voyage. Since the index patient had noted the details of his illness, he was able to answer the questions quite accurately. The passenger became ill 26 hours after boarding 10 June 2006, which is within the 12-72 hour incubation period. The index patient did not have contact with ill people during the

week before boarding the ship. He described having consumed ice cubes, but no tap water or raspberries. This interview was performed before analysis of questionnaire data, but after microbiological testing of collected food and environmental samples.

Virological Results

Stool Samples: Seventeen stool samples were received and analyzed at the RIVM (Table 3). Six of seven stool samples belonging to symptomatic and none of the 10 asymptomatic person stools tested positive for norovirus genotype GGII.4, convincingly assigning this outbreak to norovirus. Sequence analysis confirmed that the outbreak strain was a 2006b variant, which was not the same as 2006a variant strains detected during the other cruise-ship outbreak from the same company and one other ship sailing through the Netherlands at that time and for which samples were collected for testing by VWA and RIVM (Figure 2) (https://hypocrates.rivm.nl/bnwww/Divine-Event/index.html) [176].

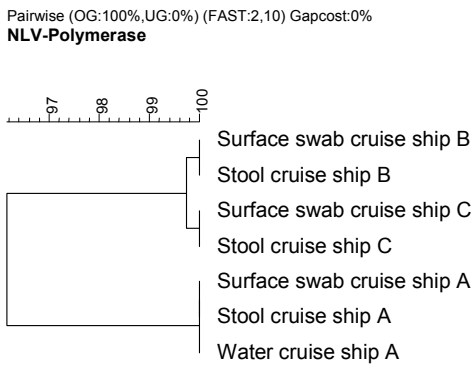

Figure 2. Norovirus strains causing outbreaks of gastroenteritis in Europe in the summer of 2006. Strains were detected in stool, water, food and surface swabs on cruise ships which sailed through the Netherlands. Cruise ship A is the cruise ship investigated in this study. Ship A and B belonged to the same ship owner.

Potential Source Samples: Eleven environmental samples were collected during the onsite investigation. Two tap water samples as well as a swab from a door handle and the toilet were taken from a cabin that belonged to a symptomatic crew member. Three swabs were taken from the handle of an alcohol-based hand-disinfection container, the restaurant door and an alcohol-disinfected elevator button. The following food samples were taken: frozen raspberries, frozen mussels, ready-to-eat tomato and cucumber salad. In five out of eleven environmental samples GGII norovirus could be detected: one of the tap waters; the toilet; the handle from a disinfection container; the restaurant door; and the raspberries. Except for the raspberries, for which no further typing was possible, in each sample the norovirus was identified as a GGII.4-2006b strain identical in an overlapping sequence of 249 nucleotides to the sequence generated from stool (Figure 2). Three weeks later tap

water was re-sampled, in which norovirus could not be detected. The ship's water supply tank included 192.10 m^3. The water quality of the drinking water at docking time in Nijmegen did not exceed the *Escherichia coli* count based European legislative standards for drinking water.

Table 3. Characteristics of the people on board and taking stool samples

Case	Crew/passenger	Origin	NLV PCR	Onset of illness	Sample date	History of illness
Yes	P	USA	+	30-06-2006	07-07-2006	No
Yes	P	USA	+	30-06-2006	06-06-2006	No
Yes	P	USA	+	30-06-2006	06-06-2006	No
Yes	P	USA	-	03-07-2006	07-07-2006	No
Yes	P	USA	+	03-07-2006	07-07-2006	No
Yes	C	Hungary	+	03-07-2006	07-07-2006	No
Yes	P	USA	+	04-07-2006	06-06-2006	No
No	C	Romania	-	n.a.	06-06-2006	No
No	C	Poland	-	n.a.	06-06-2006	No
No	C	Hungary	-	n.a.	06-06-2006	No
No	P	USA	-	n.a.	06-06-2006	No
No	C	Serbia	-	n.a.	07-07-2006	No
No	C	Romania	-	n.a.	06-06-2006	No
No	C	Germany	-	n.a.	06-06-2006	No
No	C	Germany	-	n.a.	06-06-2006	No
No	P	USA	-	n.a.	07-07-2006	No
No	P	USA	-	n.a.	06-06-2006	No

Measures Taken: No specific guidelines for control of (noro)virus outbreaks were available on board during the initial outbreak. During the previous and initial outbreak (11-25 June 2006) a set of hygiene instructions was acquired from a cleaning company at 15 June 2006. Since then, measures according to this hygiene protocol were taken accurately: public toilets were closed, patients were isolated during their illness, ill crew members disembarked, and hand washing, hygiene and disinfection measures were taken.

Discussion

Our combined epidemiological and microbiological results illustrate the difficulties of unraveling sources of infection in cruise ship norovirus outbreaks, and indicated that multiple exposures to norovirus played a role during the outbreak. Contaminated food, water, surfaces and having a sick room-mate may all have contributed to this outbreak. Proof for introduction of the virus via food or water could not be disentangled from the easily and rapidly taking over person-to-person transmission. This is a common problem during ongoing outbreaks in closed settings

and may be an important reason why cruise-related outbreaks are mostly assigned to person-to-person transmission. In order to determine whether or not a foodborne source or waterborne source is the cause of an outbreak, the initial and not a successive outbreak should be thoroughly investigated.

Since this ship was one of three ships affected by GE outbreaks and belonging to one owner, a common water- or foodborne source of infection was considered possible. This possibility was strengthened by the fact that the ships partially have the same route, menu cycle and food supplier. Our epidemiological results were based on a high response rate (93%) and indicated that contaminated tap water may have contributed to this and previous norovirus outbreaks on this river-cruise ship. The relative risk from water consumption became higher when combined with those who used ice cubes and drank juice, suggesting a potential dose-response relationship. The risk from water remained after correction for having a sick roommate. Moreover, the index case mentioned having consumed ice cubes during his incubation period on board the ship. Given that freezing is an excellent way to preserve viruses [175], and that the ice cubes were made with tap water, the consumption of water was a potential risk factor.

Food, water and environmental samples taken during the outbreak investigation tested positive. Unfortunately, however, these data could not be considered definitive proof due to the potential of cross-contamination. The positive water sample was taken from a tap in a room which was used by a symptomatic person. Consequently, contamination of the tap surface may have caused the contamination. To identify water as a cause of infection, water samples should be taken from the supply tank or a tap used by people free of symptoms. Similarly, the raspberries that tested positive were derived from an opened bag, since closed bags were not available for sampling. As crew members reported history of GE and the transmission through contaminated surfaces was clearly present, this may have resulted in contamination of the raspberries. Environmental swabs taken in public places on the ship tested positive. The norovirus positive handle of the ethanol-based hand sanitation bottle demonstrated that person-to-person transmission played a role despite – or even because of – prevention measures taken. This bottle was used for hand rubs just before having a meal at the buffet. Ethanol-based hands rubs may be effective in reducing bacterial infectivity, however, they may not be able to significantly reduce viral infectivity [293]. This situation illustrated the need for practical (noro)virus specific guidelines for both primary and secondary prevention of outbreaks on cruise ships.

However, for several reasons initial introduction via from water or food cannot be ruled out, and are points of concern for primary prevention measures. First, the tap water samples left a brownish color after filtering, suggesting suboptimal quality of the water system. Waterborne outbreaks and contamination of tap water are more often described after unusual heavy rainfall [142], which also occurred in Europe at the time of the outbreaks [180]. The cruise ship had a water tank which was filled each time the ship was docked, while using the local water system (Figure 1). The tank supply was used for consumption during sailing time. It remained unclear if disinfection of the water tank was a current procedure on this ship. In general, water tanks may be a risk for infections at cruise ships [69, 163, 269], including norovirus.

Second, raspberries are a well-described source of infection [93]. Unfortunately, the risk from consumption as garnish was not addressed in the questionnaire. Despite insufficient epidemiological evidence, the finding of norovirus-positive raspberries triggered a message in the European Rapid Alert System for Food and Feed (alert 2006.0546). There were no other illness reports associated with the product.

Attack rates for crew and passengers differed, with a significantly lower attack rate for crew, as has been described by others: four of five described outbreaks of GE on cruise ships in the United States in 2002 showed higher attack rates for passengers. A prospective survey on one of these ships showed 41% of the passengers suffering from GE, while 8% of passengers and 2% of crew sought medical attention [13]. Although an explanation was not given, the difference may be due to short-term immunity, which may have been acquired during successive outbreaks [199]. However, reporting bias could also play a role; underreporting by crew is imaginable in order not to worry the passengers or consequential loss of income, but was not addressed during our outbreak investigation. Although ill crew members were disembarked as a prevention measure during the previous outbreak, two new crew members were allowed on board shortly after their recovery of GE without the need for reporting their history of illness. This is a point of concern for the cruise ship industry, since person-to-person spread – directly or indirectly through food handling – is common in norovirus transmission. When returning to work, recovered employees need to be identified and thoroughly instructed to ensure personal hygiene, including food handling hygiene [62, 175].

Molecular analysis of viruses identified on board showed that one of the other two ships with outbreaks was contaminated with a different norovirus strain (ship B, Figure 2). On the third ship, no microbiological tests were performed for the detection of norovirus. As Figure 2 shows, strains detected on another cruise-ship (ship C) sailing through the Netherlands at that time were also distinct, although the difference was small. Interpretation of data from molecular typing needs to be undertaken with caution when no data are available about the source: in foodborne contamination events, the source of contamination is an important determinant. In food-handler associated outbreaks, typically a single strain is found and finding dissimilar sequences is supportive evidence. However, when contamination occurs higher in the food chain, e.g. during irrigation, multiple strains may be present and finding dissimilar sequences does not necessarily disprove a causal link. Only thorough outbreak investigations that include product tracing can provide definitive evidence, but this is often considered to be too complicated [110, 196].

Cruise ships are highly susceptible to norovirus outbreaks [47, 357]. Once the virus is introduced in this closed setting, person-to-person transmission plays an important role [207]. If the virus is not eliminated – either through identification of a point source, disinfection of the environment or disembarkation of shedding crew – a successive outbreak at a cruise ship is likely to occur as a consequence of a group of new and susceptible people entering the ship [256]. Therefore, the detection of point sources and the immediate implementation of accurate cleaning measures during the initial outbreak are necessary for the prevention of new outbreaks on successive trips. As cruise ships usually sail through several countries, international guidelines for reporting, investigating and controlling norovirus outbreaks on cruise ships are

needed [76]. Such guidelines need to be practicable for cruise staff, since an outbreak investigation team will mostly be present when person-to-person transmission cannot be separated from a potential point source introduction [341]. Since July, 2007, guidance for the management of cruise ships in the United Kingdom has been available online, which is a first step towards European outbreak control: http://www.hpa.org.uk/publications/2007/cruiseliners/cruiseliners.pdf.

Selection Tool for Foodborne Norovirus Outbreaks

Chapter 5

Linda P.B. Verhoef, Annelies Kroneman, Yvonne van Duynhoven, Hendriek Boshuizen, Wilfrid van Pelt, and Marion Koopmans, on behalf of the Foodborne Viruses in Europe Network.

Emerg Infect Dis 2009;15:31-8

Abstract

Detection of pathogens in the food chain is limited mainly to bacteria, and the globalization of the food industry enables international viral foodborne outbreaks to occur. Outbreaks from 2002 through 2006 recorded in a European norovirus surveillance database were investigated for virologic and epidemiologic indicators of food relatedness. The resulting validated multivariate logistic regression model comparing foodborne (n = 224) and person-to-person (n = 654) outbreaks was used to create a practical web-based tool that can be limited to epidemiologic parameters for nongenotyping countries. Non–genogroup-II.4 outbreaks, higher numbers of cases, and outbreaks in restaurants or households characterized (sensitivity = 0.80, specificity = 0.86) foodborne outbreaks and reduced the percentage of outbreaks requiring source-tracing to 31%. The selection tool enabled prospectively focused follow-up. Use of this tool is likely to improve data quality and strain typing in current surveillance systems, which is necessary for identification of potential international foodborne outbreaks.

Introduction

Globalization of the food industry, centralized production, and the wide geographic distribution of products support the need for increased international surveillance of foodborne viral outbreaks, which may occur in clusters in different countries. Because control of pathogens in the food chain requires hazard analysis critical control points and verification of measures taken, detection of the pathogen is an important step [143]. However, viral contamination of food is less likely to be recognized than bacterial contamination due to the infrequency of testing for viruses [117]. Moreover, foods acceptable by bacterial standards are not necessarily safe from viral contamination. For example, norovirus may be present in shellfish and still meet the European Union *Escherichia coli* standard for human consumption [106]. Consequently, foodborne viral infections are common, despite successful measures to reduce bacterial contamination. Recognition of foodborne viral outbreaks with international consequences would benefit from a linked and consistent reporting network among countries.

The challenge for surveillance systems is to obtain a complete dataset for the reported outbreaks [80]. The European Food Safety Authority (EFSA) recognized this challenge and began developing guidelines for an international reporting system for foodborne outbreaks caused by bacteria, viruses, or parasites (working group of foodborne outbreak surveillance, www.efsa.europa.eu). The need for a better surveillance system has also been recognized by the Foodborne Viruses in Europe (FBVE) network, which has conducted virus-specific surveillance of gastroenteritis outbreaks since 1999 [174]. Although the name FBVE suggests a foodborne focus, the network actually investigates outbreaks from all modes of transmission to obtain a comprehensive overview of viral activity in the community. A total of 13 countries are participating in the FBVE surveillance network, 11 of which are capable of collecting integrated epidemiologic and virologic surveillance data [183].

Because of the etiologic dominance of viruses, the network's primary focus is on norovirus infections [209] that have been more frequently reported in recent years after emergence of novel variant strains in the population [182, 205]. Kroneman et al. described strengths and limitations of the FBVE data collection [183] but stated that outbreak reports

need to be interpreted with caution; the number and content of these reports may vary considerably among countries because surveillance databases may be different. Most of these reports link outbreaks to person-to-person transmission; international interventions and follow-up are rare. In ≈40% of the outbreaks, no suspected mode of transmission was reported. Therefore, epidemiologic or virologic criteria should be used during the early stages of an outbreak investigation to determine whether foodborne sources should be considered. Given that surveillance systems are overwhelmed during norovirus peak seasons, use of these criteria would assist in focusing follow-up activities.

Our objective was to retrospectively derive, from surveillance data, a predictive model that could serve prospectively in the selection of norovirus outbreaks potentially related to food. Such a tool could be used to warn food safety authorities (FSAs) earlier, to improve the quality of outbreak report data, and to provide better estimates of the effects of viral foodborne disease. Our study demonstrates the added value of a reporting system amalgamated across countries; the FBVE dataset can form the basis of this tool, which may be a first step towards detection of diffuse outbreaks.

Methods

Categorizing Surveillance Systems

Because surveillance systems are known to vary in terms of design, effectiveness, and priorities [11, 161, 183, 205], a structured telephone survey/questionnaire (available from the authors) was conducted among FBVE participants to categorize national surveillance systems of the involved countries. If a participating country reported that their national system labeled outbreaks “person-to-person” as a diagnosis of exclusion, the data were excluded to avoid potential misclassification of foodborne outbreaks and consequent dilution of differentiating parameters.

Dataset

Combined epidemiologic and virologic outbreak reports from countries capable of detecting foodborne outbreaks derived from the FBVE network were collected in a protected web-based database on the basis of a structured questionnaire (www.fbve.nl/attachments/questionnaire.pdf). Collection of sequence results focused on region A of the genome (www.rivm.nl/bnwww), but allowed other entries (regions B, C, and D) because of the lack of standardization between cooperating laboratories [348]. Norovirus outbreaks were selected from this database if they fulfilled the following minimum dataset: date of onset from January 1, 2002, through December 31, 2006; norovirus detected as the only causative agent; and presence of a known norovirus sequence or genotype. The surveillance database from April 2007 was used and accounted for the median reporting lag [183] and enabled completion of data entry for outbreaks in 2006.

Parameters for Evidence

Data items used in the construction of the predictive model were derived from the EFSA draft guidelines (www.efsa.europa.eu), which are being developed to achieve consensus on the minimal variables to be reported for all foodborne outbreaks and on additional variables to be reported for thoroughly investigated foodborne outbreaks. The list has been amended with data items for foodborne outbreaks as described in comprehensive overviews [113, 311] and with data items required to enable interventions by FSA (Table 1).

Table 1. Consensus list of parameters for optimal reporting of foodborne (viral) outbreaks as defined by expert opinion, and completeness for data collected in the FBVE surveillance database

Availability in FBVE database Parameters for outbreak data	Variable	Foodborne outbreaks n=224 data (%missing)	Other mode outbreaks n=654 data (%missing)
EFSA (confirmed/probable) *			
1. Type of outbreak: general or household	Yes	224 (0)	654 (0)
2. Number of human cases†	Yes	217 (3)	651 (0)
3. Number of hospitalizations†	Yes	78 (65)	295 (55)
4. Number of deaths†	Yes	66 (70)	195 (70)
5. Foodstuff implicated	Yes	93 (58)	n.a. ‡
6. Causative agent§	Yes	224 (0)	654 (0)
7. Setting	Yes	224 (0)	654 (0)
8. Contributory factors	Yes	202 (10)	482 (26)
9. Origin of foodstuff#	*No*	*n.a. ‡*	*n.a. ‡*
10. Strength of evidence food#	*Yes*	*224 (0)*	*n.a. ‡*
EFSA (thoroughly investigated) *			
11. Reason reporting	No	n.a. ‡	n.a. ‡
12. Laboratory results food	Yes	202 (10)	n.a. ‡
13. Place food produced	No	n.a. ‡	n.a. ‡
14. Place food consumed / purchased	Descriptive	106 (52)	n.a. ‡
15. Age affected persons	Categorical	11 (95)	73 (89)
16. Gender affected persons	Yes	27 (88)	106 (84)
17. Additional information on agent	Yes	224 (0)	653 (0)
Additional parameters in literature			
18. Attack rate†	Yes	121 (46)	226 (59)
19. Seasonality	Yes	149 (33)	484 (26)
20. Duration of the outbreak†	Yes	90 (60)	265 (59)
21. Epidemic curve / point source	No	202 (10)	496 (24)
22. Sequence or variant §	Yes	224 (0)	654 (0)
23. Link with other outbreaks	Yes	22 (90)	15 (98)
Additional parameters VWA experts			
24. Incubation period	Yes	51 (77)	65 (90)
25. Illness in food handlers and their family	Partially	202 (10)	n.a. ‡
26. Presence of ill people in setting	No	n.a. ‡	n.a. ‡

* not restricted to viral; † A systematic retrospective check of Dutch data showed that variables for numbers of cases involved were reported to the national institute by regional health services when the outbreak was ongoing, and that these numbers were not updated when the outbreak had finished. The same situation was reported for other countries during the telephone survey; ‡ n.a. = not available; § inclusion criterion; # Parameters listed in italic could not be included in univariate analyses.

Definitions

Outbreaks were reported when they satisfied the agreed-upon case definition (a cluster of >2 patients within 2 days showing signs of acute gastroenteritis indicative of norovirus) [159, 174]. A gastroenteritis outbreak was ascribed to norovirus based on compatible descriptive epidemiology and laboratory confirmation according to agreed upon criteria [87]. Because norovirus outbreaks typically occur in winter, an off-seasonal period was defined as May through September; a seasonal period was defined as October through April of the following year. An outbreak was considered foodborne when infection was related to consumption of food contaminated during production or preparation. Where there was laboratory evidence of norovirus in food or analytical epidemiologic evidence for a food source through a case-control or cohort study, the outbreak was defined as confirmed foodborne. When descriptive epidemiologic data indicated a link to food, the outbreak was defined as probably foodborne. A random 50% of the total dataset was used as the training sample to build a model that distinguishes modes of transmission. The remaining 50% was used as the validation sample to validate the model. Sensitivity, or true positives, of the model for foodborne outbreaks was the proportion of the number of foodborne outbreaks correctly labeled as foodborne. Specificity, or true negatives, of the model for foodborne outbreaks was the proportion of the number of outbreaks reportedly due to person-to-person transmission that are indeed classified as person-to-person transmission. The receiver operating characteristics (ROC) curve was the graphic representation of the tradeoff between false negatives and false positives for every possible cut-off. The area under curve (AUC) was used to determine how well the predictor (based on several variables) was able to discriminate between groups (1 = perfect, 0.5 = no discrimination). Positive predictive value (PPV) was the proportion of outbreaks that met the model's foodborne criteria that are correctly labeled as such, indicating efficiency in reducing the workload of FSAs.

Data Analysis

Selected norovirus outbreaks were divided among 3 groups: confirmed or probable foodborne outbreaks; outbreaks resulting from person-to-person transmission; and outbreaks with an unknown mode of transmission. Data analysis was performed stepwise. First, completeness of data in the FBVE database with respect to the data-items in the consensus list (Table 1) was determined for outbreaks from foodborne and person-to-person transmission. Descriptive data were given for items relevant to foodborne outbreaks but not applicable to, or available for, outbreaks by person-to-person or unknown transmission. This included information concerning the food vehicle, product-handling hygiene, and place of preparation or consumption.

Second, the consensus list of predefined data items was used to compare foodborne and person-to-person outbreaks in the training sample by using logistic regression models. Variables were included in a multivariate model if they were statistically significant with $p<0.10$ during univariate analyses and if completeness of the variable was sufficient (80%) to result in a valid model. Because a logistic regression model can only be considered valid if the number of parameters is <10% of the number of outbreaks in the smallest group, analyzed variables were included as continuous where possible. The variables remained in the multivariate model if p values were <0.10, while the backward selection procedure was used or if they were found to be confounding factors for other variables in the model (β values changing at least 10%). The optimal cut-off value was determined in the training

sample and validated in the validation sample. When the validated model performed well, the β values for the final model were based on the total dataset, i.e., the validation and the training set together.

Third, the final model was used to create a web-based tool to assist public health workers in selecting outbreaks for further investigation when they receive outbreak reports, and to calculate the predicted individual probability of each outbreak with unknown mode of transmission caused by food. Individual probabilities of food relatedness were summarized to estimate the number of foodborne outbreaks in the unknown group and in the total dataset.

Results

Categorizing Surveillance Systems

Of the surveillance systems in the 13 participating countries, 11 met the FBVE network's reporting criterion of linked laboratory and epidemiologic norovirus outbreak data. Of these 11 countries, 9 were included for analysis of parameters differentiating foodborne from person-to-person outbreaks: Denmark, Finland, France, Hungary, Italy, the Netherlands, Slovenia, Spain, and Sweden. As a result of legislation, surveillance systems in 4 of these 9 countries focused on foodborne outbreaks. Six of 9 countries reported at least 1 typed outbreak per million inhabitants per year (intensive surveillance). Surveillance systems were categorized as follows: 1) intensive surveillance with focus on food; 2) intensive surveillance without focus on food; and 3) no intensive surveillance.

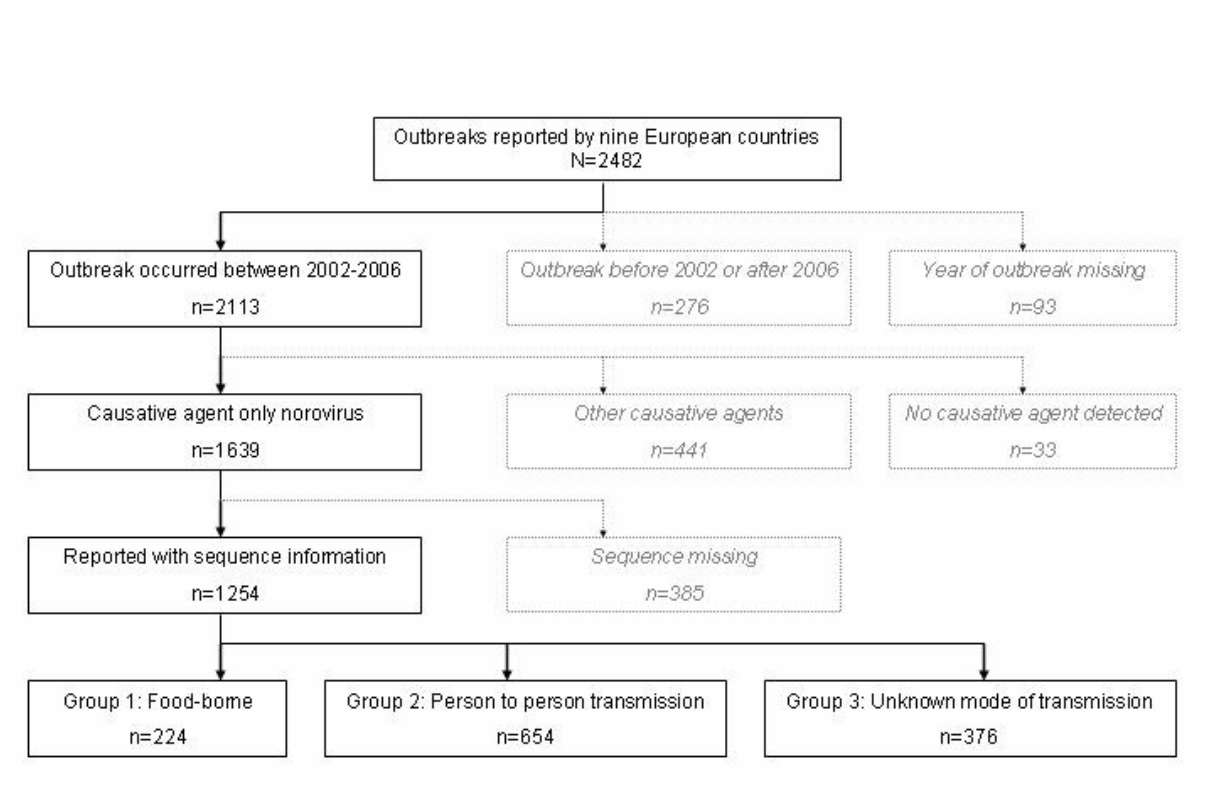

Figure 1. Outbreaks reported to the FBVE network January 2002 - December 2006, by suspected or confirmed cause and completeness of year and month of the outbreak, sequence information and mode of transmission. Other causative agents include rotavirus, hepatitis A and various bacteria.

Data Analysis

A total of 1,639 norovirus outbreaks occurring from January 1, 2002, through December 31, 2006, were reported by the countries included. Figure 1 shows the selection of the final dataset comprising 77% (1,254/1,639) of outbreaks; the remaining 23% were excluded due to missing laboratory confirmation of norovirus. Table 1 shows the completeness of analysis-set with respect to the parameters in the consensus list. The level of evidence for food-relatedness was confirmed for 24 (11%) of 224 outbreaks and was probable for 200 (89%) foodborne outbreaks. Thirty food categories were associated with outbreaks, including shellfish, fruit, fancy cakes, buffets, sandwiches, and salads. In 1 foodborne outbreak, poor personal hygiene was mentioned as a contributory factor; an infected food-handler was reported in 16 outbreaks, with 1 cook being involved in 2 outbreaks; and hygiene rather than preparation or consumption of food was mentioned in 2 outbreaks. Completeness of FBVE data with respect to the consensus list of data-items varied between items and between foodborne and person-to-person outbreaks (Table 1). Completeness of data items varied between 2% (link to other outbreaks) and 100% (type of outbreak, setting, causative agent, implicated strain). Data concerning hospitalization, attack rate, epidemic curve, incubation period, and links to other outbreaks were more likely to be reported for foodborne outbreaks.

On the other hand, seasonality and contributory factors were more frequently reported for outbreaks with other modes of transmission. The items in italics in Table 1 could not be included during univariate analyses because they played a role only when foodborne transmission occurred or because data were not requested in the FBVE surveillance system. Risk factors resulting from univariate analyses are presented in Table 2 and show that foodborne outbreaks were found more often in households or restaurants and less often in healthcare settings, involved nongenogroup (GG) II.4 strains relatively more frequently, were more likely to occur during off-seasonal months, and involved more cases when notified compared to outbreaks from person-to-person transmission. Table 2 shows all parameters included in the multivariate analysis that remained in the model during the backward selection procedure. The AUC in the training sample and in the validation sample was 0.92 and 0.90, respectively, indicating the model performs very well in distinguishing foodborne outbreaks from person-to-person transmission (Figure 2). In the validation sample, the optimal cut-off value (the value of the ROC curve closest to the upper left corner) resulted in a sensitivity of 0.72, a specificity of 0.92, and a PPV of 0.64; a follow-up of outbreaks would have focused on 24% of the total number of reported outbreaks. The probability that an outbreak was attributed to food was calculated by using the following final model, based on the complete dataset of 878 records and corrected for characteristics of national surveillance systems:

$$\text{Odds (foodborne = 1)} = \frac{P(foodborne = 1)}{P(foodborne = 0)}$$

= exp (1.5477 + 0.8065 when in household)
+ (0.0322 for each involved case when notified)
+ [(3.0999 when in restaurant) or – (1.2963 when in hotel) or – (2.6616 when in hospital) or – (1.9912 when in daycare) or – (0.5289 when in school)]
+ (0.3190 when GGnonII.4)
– [(1.0270 if intensive surveillance and focus food) or – (2.0540 if intensive surveillance and no focus food)]

Table 2. Factors (8 of 17) (borderline) significant during univariate logistic regression in a random selection of 50% of the dataset for comparison of foodborne outbreaks (group 1) and outbreaks from other modes of transmission (group 2)

Indicator	Category/measure	Group 1 (n=112)	Group 2 (n=327)	Univariate OR (95%CI)	Univariate adjusted for country OR (95%CI)	Multivariate adjusted for country OR (95%CI)
1	General	105	325	Reference	Reference	Reference
	Household	7	2	10.8 (2.2-52.9)	10.1 (1.6-64.3)	0.1 (0.0-1.0)
2	Number of cases*	-	-	1.1 (1.0-1.1)	1.0 (0.9-1.1)	1.1 (1.0-1.2)
7	Residence	7	2	Reference	Reference	Reference
	Restaurant	36	1	10.3 (0.8-129.4)	13.2 (0.7-234.0)	>999†
	Healthcare institute	27	267	0.0 (0.0-0.1)	0.0 (0.0-0.1)	0.0 (0.0-0.0)
	Day-care	2	15	0.0 (0.0-0.3)	0.1 (0.0-0.9)	0.0 (0.0-0.1)
	Hotel/ guest house	9	12	0.2 (0.0-1.3)	0.1 (0.0-1.3)	0.0 (0.0-0.1)
	School	11	9	0.3 (0.1-2.1)	0.3 (0.0-2.7)	0.0 (0.0-0.2)
	Other	20	21	0.3 (0.1-1.5)	0.3 (0.0-2.1)	0.0 (0.0-0.2)
17	Non-GGII.4	55	48	Reference	Reference	Reference
	Genogroup II.4	57	278	0.2 (0.1-0.3)	0.2 (0.1-0.4)	0.4 (0.2-1.0)
18	*Attack rate**	-	-	*14.0 (3.6-54.0)*	*6.7 (1.5-34.3)*	-
19	May-Sept	20	35	Reference	Reference	-
	Oct-April	47	208	0.4 (0.2-0.7)	0.5 (0.3-1.2)	
20	Duration in hours*	-	-	0.9 (0.8-0.9)	0.9 (0.8-1.0)	-
21	*No point source*	*60*	*242*	*Reference*	*Reference*	-
	Point source	*43*	*3*	*58.8 (17.3-192.7)*	*44.7 (11.8-167.7)*	

Significant factors were included in multivariate analyses to construct the final model, –, entered as a continuous variable. Parameters in italic could not be included in multivariate analysis because of missing values * A systematic retrospective check of Dutch data showed that variables for numbers of cases involved were reported to the national institute by regional health services when the outbreak was ongoing, and that these numbers were not updated when the outbreak had finished. The same situation was reported for other countries during the telephone survey; † The parameter restaurant was set to 0 because the variable was a linear combination of other variables as follows: Restaurant = intercept – household – health care – day care – hotel – school – other > 99.

This final model (sensitivity = 0.80, specificity = 0.86, PPV = 0.65) can be prospectively applied to calculate potential food-relatedness of reported norovirus outbreaks and reduce the number of outbreaks to 31% of all reported outbreaks. The practical web-based tool created with the model can be found in the online Technical Appendix, available from www.cdc.gov/EID/content/15/1/31-Techapp.xls, of which the image is shown in Figure 3. If this tool is used by a nongenotyping country, intensive surveillance can be considered to be at least 2 reported outbreaks (instead of 1 typed outbreak) per 1 million inhabitants per year. For the nongenotyping countries, the unknown genotype in the model resulted in an additional 5 unrecognized foodborne outbreaks coexisting with a slight reduction of sensitivity (0.78), equal specificity (0.86), PPV (0.65), and 30% of the outbreaks requiring follow-up. Of 376 outbreaks with an unknown mode of transmission, data for 352 (94%) were sufficient to calculate the probability of a foodborne outbreak. Summarizing individual probabilities resulted in 100 (29%) of 352 potential foodborne outbreaks in the unknown group; summarizing probabilities in the total dataset resulted in an estimated 280 (22%) of 1,254 reported outbreaks being possibly foodborne.

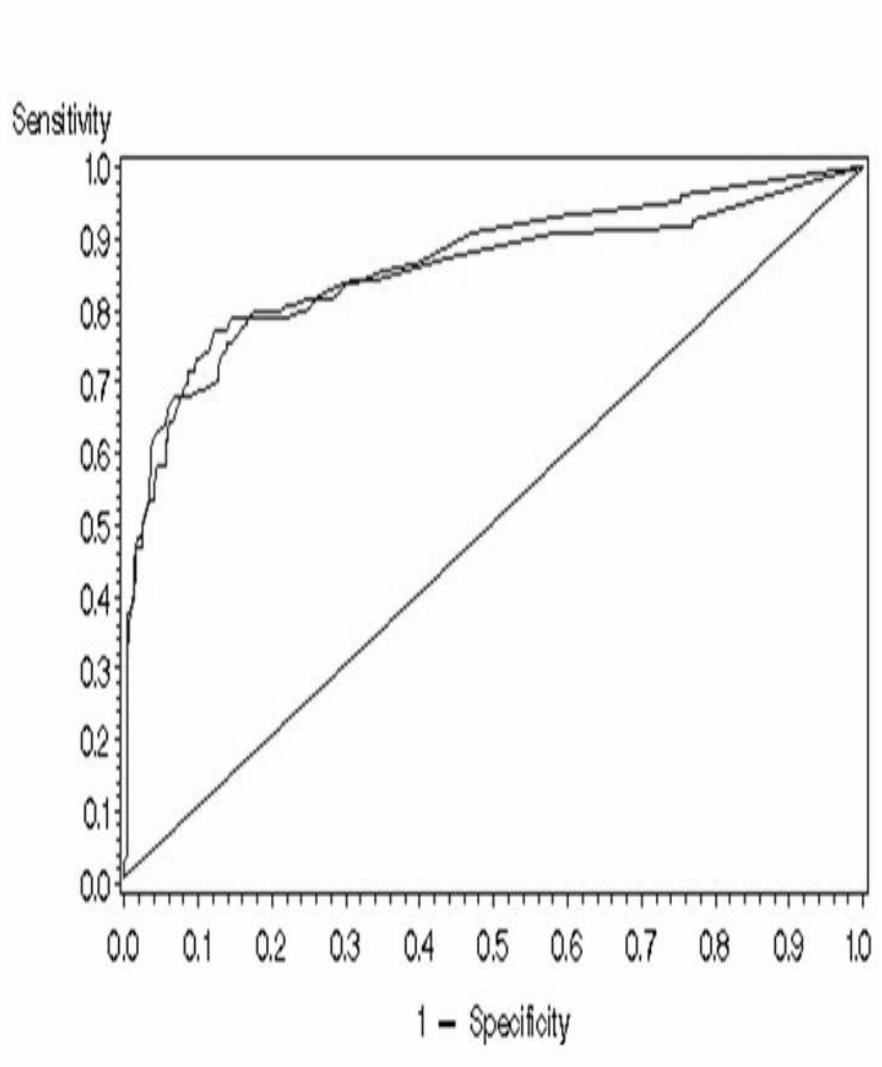

Figure 2. Receiving operator characteristics curves for distinction of foodborne outbreaks from person-to-person outbreaks in the training sample (upper graph, 435/439 records used) and in the validation sample (lower graph, 432/439 records used). The area under the curve in the validation sample was 0.90, indicating good performance of the model.

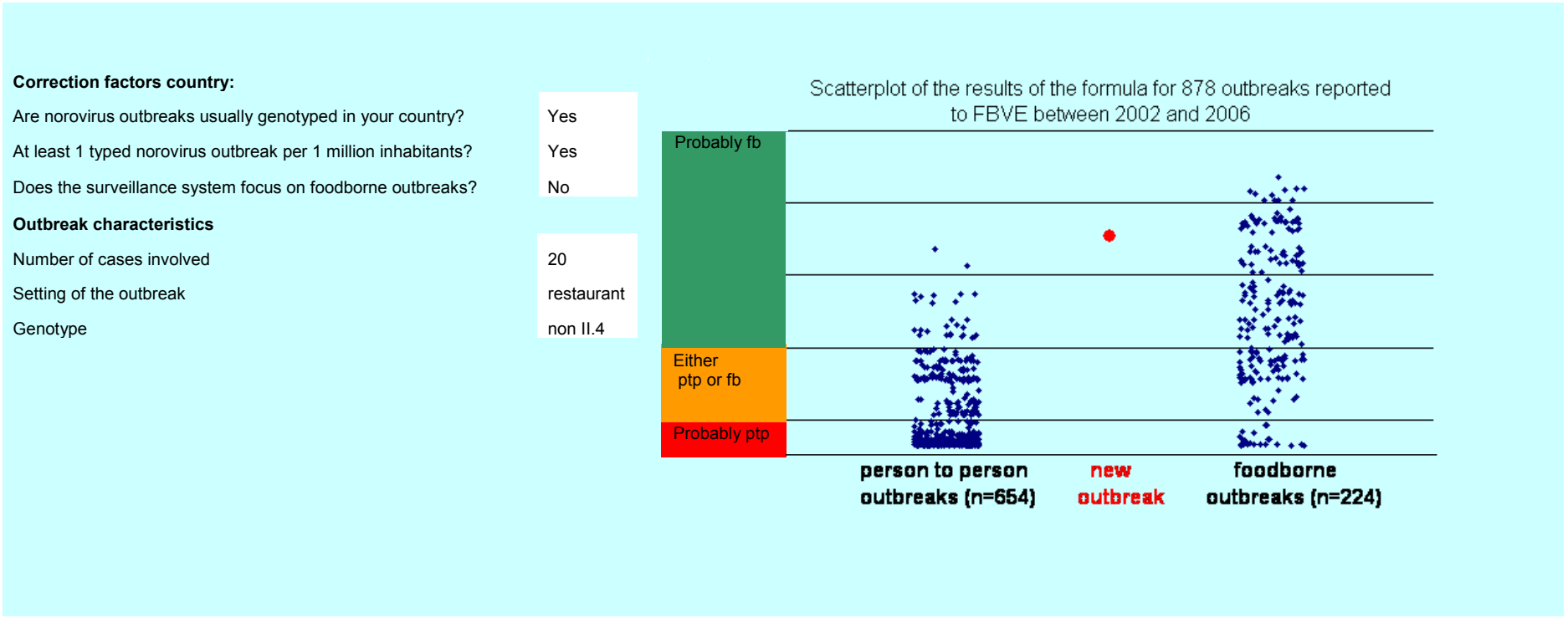

Figure 3. An example of the typing tool available from www.cdc.gov/EID/content/15/1/31-Techapp.xls. At the time a national public health institution is notified of an outbreak this tool can be used with the minimum data available at that time. If the requested data are not (yet) available, the data field stays empty. Step 1: choose the characteristics of the surveillance system in your country, i.e. presence of genotyping of reported outbreaks, intensity of norovirus surveillance, a foodborne focus as a result of legislation. Step 2: enter the requested outbreak characteristics, i.e. number of cases (as far as known at the reporting moment), setting in which the outbreak took place, genotype (if available).

Discussion

We built and validated a model to estimate the likelihood that a norovirus outbreak was related to food. This study was the basis for a practical tool that can prospectively be applied in near real-time in the European setting to identify potential foodborne viral outbreaks in both genotyping and nongenotyping countries. The model can also retrospectively estimate the true contribution of food to norovirus outbreaks in Europe, and may contribute to studies estimating the effects of foodborne gastroenteritis. Moreover, the user-friendly tool may support more consistent reporting and typing of viral outbreaks. Our approach is innovative for norovirus surveillance and provides a new estimate for the proportion of foodborne outbreaks that is higher (22%) than the recognized proportion of foodborne outbreaks in countries that can separate transmission modes (18%). However, this higher estimate is based on reported outbreaks and therefore does not account for underreporting or overreporting.

Using this selection mechanism prospectively for identification of outbreaks requiring detailed follow-up, FSAs can focus on 31% of all reported outbreaks and accept that 1 of 5 foodborne outbreaks will be missed. This finding may appear to lack sensitivity, but at present only a few foodborne outbreaks are investigated sufficiently to provide information on the basis of which FSAs can act, which we will illustrate. In 2007, a year when an unusually high number of norovirus outbreaks were reported, 1 of the 37 Municipal Health Service agencies in the Netherlands reported a total of 28 norovirus outbreaks. Applying this model to reduce the number of outbreaks needing investigation from 28 to 9 is likely to greatly improve the potential for intervention and the quality of surveillance data for foodborne outbreaks. The tool, based on this model, will be implemented and evaluated in the Netherlands in 2010-2011.

As previously identified, restaurants were the most common setting for foodborne outbreaks [131, 250]. Our model output, however, also provides a strong first indication that the epidemiology of norovirus outbreaks differs between genotypes because the proportion of non-GGII.4 outbreaks was higher in foodborne outbreaks. Non-GGII.4 outbreaks indicated source contamination, altering the probability of outbreaks being related to food (online Technical Appendix, example in Figure 3). Unfortunately, many countries cannot take advantage of this result because genotyping is not among their routine procedures. For this reason, the practical tool was adjusted so that it can be restricted to epidemiologic parameters only. However, a rapid assay should be developed that discriminates GGII.4 from non-GGII.4, which would enable earlier and more targeted measures by FSAs on a large scale.

The difference identified between GGII.4 and non-GGII.4 is a first step towards identification of international foodborne outbreaks, of which some examples are known [93, 169, 353]. Detailed strain type and sequence information may provide the linking conditions for such outbreaks. Unfortunately, analysis of strain types did not give statistically significant results but did suggest the existence of differences, which should be separately investigated. More data are needed about the diversity of noroviruses belonging to rare genotypes to reliably use the data when identifying a probable source of infection. This diversity is illustrated through a recent example. In the spring of 2006, an unusually high number of norovirus outbreaks was reported that involved passengers on cruise ships in European waters [176]. The finding that several of the outbreaks were caused by a distinct strain of GGII.4 norovirus triggered an outbreak investigation which tested the hypothesis that these

outbreaks might have resulted from a common source [341]. More detailed molecular characterization, outbreak investigations, and use of molecular strain data from surveillance of land-based outbreaks showed that the new variant strain viruses could be found across Europe, thus reflecting a widespread epidemic rather than a common source event. Much less is known about noroviruses belonging to the rare genotypes. For instance, if these viruses behave in an opportunistic fashion, they will circulate in the community without causing outbreaks, going undetected because routine surveillance for sporadic cases is rare [370]. Until further investigation can show epidemiologic characteristics of rare genotypes, the selection tool using information on setting, genogroup, and number of cases enables quick screening of outbreaks of interest.

Several countries have conducted studies using methods to estimate the proportion of foodborne gastroenteritis [102, 229, 359]. These studies have identified foodborne norovirus infections varying from 1/33 inhabitants in the United States to 1/780 in the United Kingdom. Prospective cohort studies are usually the most accurate method of ascribing illness to food, but are costly. Deriving estimates from existing data collections has its weaknesses [262], but the data collections available provide a good tradeoff between costs and providing useful information for public health. Numbers based on surveillance data need to be interpreted and extrapolated with caution, as international differences in surveillance systems can introduce bias.

Although our approach is innovative in categorizing norovirus outbreaks in a surveillance system, it is commonly applied in medical research to predict critical diagnostic outcomes [90, 145, 316]. A limitation of our data is that selection of outbreaks may have occurred in the database, e.g., if outbreaks were not reported until they had reached a certain size because of (secondary) person-to-person transmission [116]. Conversely, outbreaks likely to be foodborne may have an origin other than food [221], and confirmation of this is rare. In addition, the definition applied for a foodborne outbreak may differ substantially among countries. We reduced the chance of misclassification by using retrospectively applied uniform definitions, and by selecting those countries clearly discriminating transmission modes. During the survey for categorization of surveillance systems, we confirmed that proof of a foodborne outbreak is often difficult to obtain; the transmission mode consequently may remain unknown relatively more frequently than that of person-to-person outbreaks. However, the slight difference between the foodborne proportion of outbreaks among the outbreak with unknown (28%) and known (26%) transmission does not suggest a difference in the estimated and reported proportion.

The European norovirus surveillance system, like most surveillance systems, has to cope with reporting delays and missing values [11, 80, 183]. Because virologic and epidemiologic distinctive parameters were of interest, strict selection criteria were used in this study. Incompleteness in our selection criteria left us with 1,254 (66%) of 1,639 norovirus outbreaks in our analysis dataset. Greater completeness of our analysis dataset may have resulted in an extended model that included a larger variety of indicators as proposed by EFSA, which requested an extensive minimal dataset for foodborne outbreaks from differing causes. However, our model was able to distinguish norovirus foodborne outbreaks with far fewer indicators than those prescribed by EFSA. Because an optimal surveillance system for detection of diffuse foodborne outbreaks is dependent on completeness of a minimal dataset, use of the tool is likely to be a first step towards such a system.

We developed a practical tool that can distinguish food-relatedness of norovirus outbreaks and is likely to improve surveillance data quality. A model that predicts foodborne outbreaks regardless of causative agents, and that links conditions for viral outbreak, should be the focus of future studies. The requested minimum dataset for surveillance of foodborne outbreaks with potential for international consequences needs to be clearly defined. The more information needed, the less the compliance; priority should therefore be given to information essential for initiating interventions.

Use of Norovirus Genotype Profiles to Differentiate Origins of Foodborne Outbreaks

Chapter 6

Linda Verhoef, Harry Vennema, Wilfrid van Pelt, David Lees, Hendriek Boshuizen, Kathleen Henshilwood, and Marion Koopmans, on behalf of the Foodborne Viruses in Europe network.

Emerg Infect Dis 2010;16:617-24

Abstract

Because secondary transmission masks the connection between sources and outbreaks, estimating the proportion of foodborne norovirus infections is difficult. We studied whether norovirus genotype frequency distributions (genotype profiles) can enhance detection of the sources of foodborne outbreaks. Control measures differ substantially; therefore, differentiating this transmission mode from person-borne or food handler–borne outbreaks is of public health interest. Comparison of bivalve mollusks collected during monitoring (n = 295) and outbreak surveillance strains (n = 2,858) showed 2 distinguishable genotype profiles in 1) human feces and 2) source-contaminated food and bivalve mollusks; genotypes I.2 and I.4 were more frequently detected in foodborne outbreaks. Overall, ≈21% of all outbreaks was foodborne; further analysis showed that 25% of the outbreaks reported as food handler–associated were probably caused by source contamination of the food.

Introduction

Noroviruses are members of the family *Caliciviridae* and recognized as major pathogens in outbreaks of gastroenteritis worldwide. Because these viruses have environmental stability [45], ability to use different transmission routes, and low infective doses [77], their source may be difficult to determine during an outbreak. Transmission can occur through contact with shedding persons; food contaminated during processing, preparation or serving; sewage-contaminated water used for consumption, cultivation or irrigation of food; contaminated aerosols resulting from vomiting; and environmental contamination [18, 86] Five genogroups have been described (GI–V), subdivided into at least 40 genetic clusters [120, 174].

To implement effective measures for prevention, recognition of the transmission routes is necessary. Consequently, the relative importance of different transmission routes in the total number of outbreaks is of interest for estimation of cost-effectiveness of reducing the number and size of norovirus outbreaks, particularly for geographically disseminated foodborne outbreaks. Such outbreaks are difficult to detect when the primary introduction of viruses through food occurs simultaneously in several countries or continents [93, 196, 353]. Globalization of the food industry with consequential international distribution of products increases the risk for such outbreaks. For example, the first reported GII.b outbreak occurred in August 2000 during a large waterborne outbreak in southern France [107]. After this outbreak, in December and January, 4 multi-pathogen and oyster-related outbreaks with this newly emerging genotype were reported from France. In the same period, Denmark, Finland, and the Netherlands reported norovirus cases resulting from oysters originating from a French batch that probably was sold in these countries, as well as in Sweden, Italy, and Belgium [174]. All these outbreaks seemed to involve closely related and newly detected GII.b strains. After active case identification, further linked cases were detected in Germany, the United Kingdom, Spain, Slovenia, and Sweden [40, 171]. Another example of a geographically disseminated outbreak was several seemingly independent norovirus outbreaks in Denmark that were traced back to consumption of raspberries from Poland. Although raspberries from this contaminated batch were exported to other European countries, an alert in the Rapid Alert System for Food and Feed did not result in further linked outbreak reports [93]. Thus, geographically disseminated outbreaks are sometimes identified

but only after the joint and exhaustive efforts of different organizations, such as laboratory networks, food safety authorities, and public health institutions. Knowledge of the proportion of geographically disseminated foodborne outbreaks to all norovirus outbreaks will therefore provide insight into the cost-effectiveness of such efforts.

We studied whether the genotype frequency distributions (genotype profiles) of strains can be used to differentiate foodborne outbreaks related to contamination early in the food chain (i.e., during primary production) from those related to contamination later in the food chain (i.e., during preparation or serving). If so, detection of food origins likely to cause geographically disseminated outbreaks will be enhanced. We considered methods for attribution to multiple sources commonly applied to Salmonella infections [124] because different transmission routes involved in norovirus infections can disguise the foodborne origin. However, such methods require strain collections representative of noroviruses in the potential sources that are as yet unavailable because of difficulties in the direct detection of viruses in food [33, 194, 285]. Therefore, we compared 2 strain collections: noroviruses identified through filter-feeding bivalve mollusk monitoring representing source contamination of food and noroviruses collected through systematic surveillance of illness in the population. The first was collected by the European Community Reference Laboratory for Monitoring Bacteriological and Viral Contamination of Bivalve Mollusks during 1995–2004 [118] and the second by the Foodborne Viruses in Europe (FBVE) network, which has conducted surveillance for norovirus outbreaks in Europe since 1999. Prior investigation of the FBVE database of systematically collected epidemiologic and microbiological norovirus surveillance data [174] showed that the epidemiology of norovirus outbreaks in Europe varies between genogroups. An analysis of the properties of reported outbreaks indicated a clear difference between GII.4 strains and other noroviruses; non-GII.4 strains were found more frequently in outbreaks with a foodborne mode of transmission, and GII.4 strains were found more frequently in healthcare settings with person-to-person transmission [184, 343]. Here we demonstrate that further specification into genotypes shows additional differences in the epidemiology of norovirus outbreaks.

Methods

Data Sources

We used 2 broad databases reflecting norovirus prevalence within the European countries under surveillance. These databases provided us the opportunity to compare genotype proportions as detected in outbreaks, i.e., human surveillance data, with those detected in source-contaminated food products, i.e., bivalve mollusks monitoring data.

Human Surveillance Data

From January 1999 through December 2004, FBVE collected molecular information on 2,727 norovirus outbreaks and sporadic cases in Denmark, Finland, France, Germany, England and Wales, Hungary, Ireland, Italy, the Netherlands, Norway, Sweden, Slovenia, and Spain [183, 209]. Although the name FBVE suggested a foodborne focus, the network actually investigated outbreaks from all modes of transmission to obtain a comprehensive overview of viral activity in the community (strengths and limitations of the FBVE data collection were described by Kroneman et al. [183]; to compare newly detected strains with the FBVE database and find potential linked outbreaks, we used a comparison tool [www.rivm.nl/bnwww]). Data were reported to FBVE at outbreak level; therefore, no informed

consent was needed. Outbreaks were categorized as follows on the basis of the cause of infection as reported in the surveillance system:

- Foodborne-food (FB-food) when an outbreak was reported to be caused by food and the outbreak strain was detected in food;
- Foodborne-feces (FB-feces) when an outbreak was reported to be caused by food and the outbreak strain was detected in human feces only;
- Foodborne (FB) when an outbreak was classified as FB-food or FB-feces;
- Food handler–borne (FHB) when an outbreak was reported to be caused by an infected foodhandler contaminating the food and the outbreak strain was detected in human feces;
- Person-borne (PB) when an outbreak was reported to be caused by person-to-person transmission and the outbreak strain was detected in human feces;
- Unknown (UN) when the mode of transmission was not reported or was reported to be unknown and the outbreak strain was detected in human feces.

When the mode of transmission was not reported but information was given in text data fields, this information was used to categorize the outbreak. Because we were interested in the origin of the virus, we categorized outbreaks involving PB transmission but starting with food as FB-food, FB-feces, or FHB, depending on available information. Strains detected in sporadic cases were clustered into outbreaks if information was available. The remaining strains detected in sporadic cases were considered of interest with respect to the genotypes causing human illness and representative of potential unreported outbreaks. When we detected multiple genotypes during an outbreak or in sporadic cases, we recorded each genotype.

Bivalve Mollusk Monitoring Data

The European Community Reference Laboratory for Monitoring Bacteriological and Viral Contamination of Bivalve Mollusks systematically collected sequence data on norovirus strains routinely detected in bivalve mollusks in Europe. During January 1999–December 2004, the laboratory systematically collected 295 strain sequences with region A sequence lengths varying from 76 to 78 nt. These strain sequences were detected as part of production area monitoring studies or outbreak investigations of gastroenteritis in Denmark, England and Wales, Ireland, Scotland, and Spain. All samples were first routinely tested with GI and GII PCR methods; then all positive samples were cloned [133], resulting in a representative reflection of norovirus presence in bivalve shellfish. If we detected multiple genotypes in 1 sample, we recorded each genotype.

Assignment of Genotypes

Strains were genotyped by using a previously described method for sequence analysis of a fragment of the RNA-dependent RNA polymerase gene regions B, C, and D [346] because these regions were used in the FBVE network. From the start, the network used sequence-based genotyping of the then most commonly used diagnostic PCR fragment, targeting the RNA-dependent RNA polymerase gene. Since then, however, it has become clear that recombination is common and mainly occurs in the area between the overlap between the polymerase and the capsid gene. Therefore, capsid-based and polymerase-based typing may be discordant. Genotype assignment was therefore performed only after

clustering of query strains against all relevant available sequences in the FBVE database (Kroneman et al., unpub. data). This process resulted in the genotyping of all but 68 (2%) strains. Genotypes were classified on the basis of their similarity to reference strains representing known genotypes by using the norovirus typing library (www.noronet.nl/nov_quicktyping). If the (clustered) genotypes occurred <5 times in our 5-year covering data selection, the frequencies were considered too low to be ascribed a separate genotype and excluded. This was the situation for GII.18 and 6 clusters of nonassigned GII strains (n = 25, 1%).

Data Analysis

First, we compared the genotype frequency distributions detected in outbreak categories reported as FB-food, FB-feces, FHB, PB, and UN and in routinely tested bivalve mollusks. To evaluate the correlation and measures of association of these 6 proportional profiles, Pearson correlation coefficient ρ was calculated on the basis of frequencies (ρ_1) and logarithm (ρ_2) of the frequencies of 22 genotypes, as well as Cramer V and simulated p values by using 20,000 replications with the exact variant of the χ^2 test. The exploratory technique correspondence analysis allows for examining the structure of categorical variables in a multiway table and was used to visualize the measure of correspondence in the 6 genotype profiles. p values <0.05 were considered significant.

Second, to differentiate the remaining genotype profiles detected in outbreaks, we used the genotype profiles of the 2 main transmission modes to be distinguished during an outbreak investigation. For each genotype in the human surveillance collection, the fraction of outbreaks of known origin being FB (i.e., FB-food and FB-feces) or PB was estimated on the basis of the proportion of FB outbreaks of all FB + PB outbreaks in each genotype. We used the estimated proportion of FB outbreaks of all FB + PB outbreaks in each genotype to estimate the probability that an FHB or UN outbreak was foodborne. We calculated 95% confidence intervals (CIs) using Monte Carlo simulation with 10,000 random draws from the β distributions, which are the posterior probabilities of the proportions [37].

Results

Of 3,022 detected noroviruses, 25 (1%) were excluded because of low frequencies; for 68 (2%), assignment of a genotype was not possible because of short sequences or inability of the method applied to type the detected norovirus beyond its genogroup. Of the remaining 2,929 strains, 71 (2%) could not be linked to epidemiologic data, and therefore their origin remained unknown, leaving 2,858 (95%) strains for analysis: 922 originating from PB outbreaks, 24 from FB-food outbreaks, 151 FB-feces outbreaks, 20 FHB outbreaks, 1,446 UN outbreaks, and all 295 bivalve mollusk monitoring strains. Among the outbreaks of known origin, 175 (16%) of 1,117 were reported to be FB (i.e., FB-food and FB-feces).

The proportion of genogroup I was significantly higher in bivalve mollusks (137/295, 46%) than in infected humans (313/2,539, 12%) (Table 1). All genotypes detected in bivalve mollusks were also detected in humans; however, 9 genotypes causing human illness were not detected in bivalve mollusks. Overall, the II.4 genotype was responsible for most of the human outbreaks (1,326/2,539, 52%), followed by II.b (328/2,539, 13%) and II.7 (156/2,539, 6%).

Table 1. Number of norovirus strains detected in samples from humans, bivalve mollusks and food, specified by norovirus genotypes*

	Genotypes		Human surveillance, no. strains					Bivalve monitoring, no. strains	Total no. strains
	Pol-based	Cap-based	FB-food	FB-feces	FHB	PB	UN	BM	
Genogroups	I.1	I.1	1	8	0	5	18	0	32
	I.2		0	6	0	1	32	8	47
	I.3	I.3	0	8	3	16	80	13	120
	I.4	I.4	9	8	1	8	46	86	158
	I.5		0	0	0	1	5	3	9
	I.6	I.6	2	3	1	21	17	25	69
	I.7		0	1	0	0	7	2	10
	NA I.a	NA I.a	0	1	0	0	4	0	5
	II.1		0	5	2	12	94	7	120
	II.2	II.2	0	13	1	27	66	0	107
	II.3		0	1	0	1	38	11	51
	II.3R	II.3	0	1	0	1	41	2	45
	II.4	II.4	5	47	9	681	584	63	1389
	II.5		0	3	0	6	12	0	21
	II.8		1	0	1	1	13	0	16
	NA II.a		0	0	0	2	7	0	9
	NA II.c		0	2	0	8	31	1	42
	NA II.d		0	1	0	3	8	0	12
	IV.1		0	2	0	1	8	0	11
Recombinants	NA II.b	II.1, II.2, II.3	4	23	1	100	200	63	391
	II.1	II.10	0	0	0	8	19	11	38
	II.7	II.6, II.7	2	18	1	19	116	0	156
Total			24	151	20	922	1446	295	2858

*pol, polymerase; cap, capsid; FB-food, foodborne-food, i.e. an outbreak was reported to be caused by food and the outbreak strain was detected in food; FB-feces, foodborne-feces, i.e. an outbreak was reported to be caused by food and the outbreak strain was detected in human feces; FHB, food handler-borne, i.e. an outbreak was reported to be caused by an infected food handler contaminating the food and the outbreak strain was detected in human feces; PB, person-borne, i.e. an outbreak was reported to be caused by person-to-person transmission and the outbreak strain was detected in human feces; UN, unknown, i.e. the mode of transmission was not reported or was reported to be unknown and the outbreak strain was detected in human feces.

We visualized genotype frequency distributions as profiles for the observed categories of outbreaks and sorted them for their relevance in UN outbreaks, presented with different scales allowing for proportional comparison (Figure 1). The genotype profiles vary between these groups. The correlation coefficients based on frequencies, ρ1, showed that 2 genotype profiles were distinguishable (Table 2): 1 profile typically seen in human feces (FB-feces, FHB, or PB), and another profile typically detected in sources other than human feces, i.e., in food (FB-food) or bivalve mollusks. The ρ1 reflects some genotypes frequently and others rarely seen in FB-food and bivalve mollusks. Because FB-food strains include oyster-related outbreaks as well, we assumed that the correlation between FB-food and bivalve mollusks can be explained partly by these oyster-related outbreaks. We therefore calculated an additional correlation coefficient using the 14 strains detected in food items other than bivalve

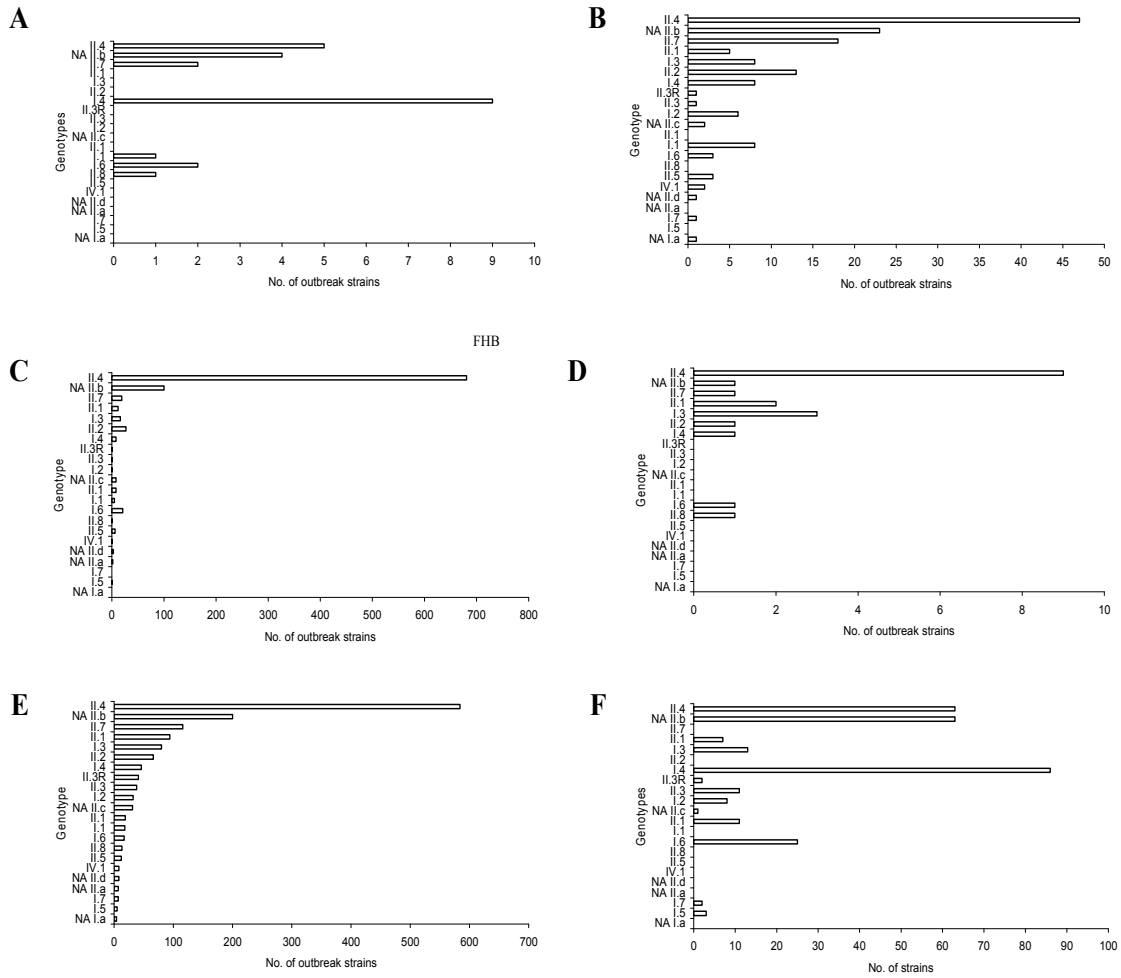

Figure 1. Norovirus genotype profiles detected in foodborne outbreaks, showing strains detected in A) foodborne-food, i.e. an outbreak was reported to be caused by food and the outbreak strain was detected in food; B) foodborne-feces, i.e. an outbreak was reported to be caused by food and the outbreak strain was detected in human feces; C) person-borne outbreaks i.e. an outbreak was reported to be caused by person-to-person transmission and the outbreak strain was detected in human feces;; D) food handler–borne outbreaks, i.e. an outbreak was reported to be caused by an infected food handler contaminating the food and the outbreak strain was detected in human feces; E) outbreaks with an unknown mode of transmission; and F) routine monitored bivalve mollusks. The genotypes are sorted for their relevance in unknown outbreaks.

mollusks. Despite low numbers, this calculation resulted in a high, significant correlation coefficient ($\rho = 0.81$, $p < 0.001$). The logarithm of the frequencies, ρ2 (Table 2), is less sensitive to peak frequencies of genotypes and therefore capable of differentiating profiles with respect to the rare genotypes and approaching the Cramer V. Cramer V and ρ2 show less clear association of profiles, with diverging results for the FHB and UN profiles. Table 2 shows the quantification of association; the associated genotype profiles illustrated by correspondence analysis are shown in Figure 2. The values of the 6 columns in Table 1 can be considered coordinates in a 6-dimensional space, and the distances are computed. These distances summarize information about the similarity between the rows in Table 1. Dimension 1 may be considered to differentiate transmission modes explaining 59.12% of the correspondence, confirming that the profiles found in bivalve mollusks and FB-food are

similar with regard to the pattern of relative frequencies in genotypes (rows in Table 1) and differ from those in PB. It also shows that the FHB, UN, and FB-feces profiles are mutually similar, with their distance somewhere between the PB and FB-food/bivalve mollusk profiles. Dimension 2 may represent dual origin, explaining an additional 31.40%, showing that FB-feces, FHB, and UN mutually correspond and differ from FB-food, bivalve mollusks, and PB that mutually correspond.

When we compared the proportions of genotypes detected in FB outbreaks with those in PB outbreaks, we detected genotypes I.2 and I.4 significantly more frequently in FB outbreaks (Figure 3). On the other hand, genotypes I.6, II.1-recombinant, II.2, II.4, II.b, II.c, and II.d were detected significantly more frequently in PB outbreaks. Using these proportional FB and PB genotype profiles and their confidence intervals to distinguish between FB and PB transmission among 20 FHB outbreaks, we could ascribe 5 (95% CI 4–6) to FB and 15 (95% CI 14–16) to PB transmission. Ascribing 1,446 unexplained human norovirus outbreaks to either FB or PB transmission resulted in ≈367 (95% CI 327–417) FB outbreaks and ≈1,079 (95% CI 1,026–1,120) PB outbreaks. Overall, use of the genotype patterns increases the estimated number of FB proportion of outbreaks to 21% (547/2,563; range 20%–23%) compared with the 16% previously mentioned among the outbreaks of known origin.

Table 2. ρ_1, ρ_2 and Cramer V with simulated p-values (20,000 replications) of 6 norovirus genotype patterns as detected in routinely tested bivalve shellfish, and during norovirus outbreaks, 1999-2004*

Source		FB-food (p value)	FB-feces (p value)	FHB (p value)	PB (p value)	UN (p value)	BM (p value)
FB-food	ρ_1	1.00	0.48 (0.02)	0.40 (0.07)	0.43(0.04)	0.48(0.02)	0.91(<0.01)
	ρ_2		0.46 (0.03)	-0.15 (0.51)	0.34 (0.12)	0.24 (0.26)	0.47 (0.03)
	V		0.47 (0.01)	0.62 (0.04)	0.48 (<0.01)	0.26 (<0.01)	0.41 (0.02)
FB-feces	ρ_1		1.00	0.93(<0.01)	0.92(<0.01)	0.96(<0.01)	0.53 (0.01)
	ρ_2			0.40 (0.06)	0.55 (<0.01)	0.66 (<0.01)	0.69 (<0.01)
	V			0.34 (0.41)	0.43 (<0.01)	0.19 (<0.01)	0.57 (<0.01)
FHB	ρ_1			1.00	0.97(<0.01)	0.93(<0.01)	0.43(<0.05)
	ρ_2				0.22 (0.32)	0.39 (0.07)	0.47 (0.03)
	V				0.25 (<0.01)	0.09 (0.75)	0.46 (<0.01)
PB	ρ_1				1.00	0.96(<0.01)	0.51(0.01)
	ρ_2					0.61 (<0.01)	0.53 (0.01)
	V					0.42 (<0.01)	0.63 (0.01)
UN	ρ_1					1.00	0.59(<0.01)
	ρ_2						0.65 (<0.01)
	V						0.48 (<0.01)
BM							1.00

* ρ_1 = Pearson correlation coefficient based on frequencies; ρ_2 = Pearson correlation coefficient based on logarithm of frequencies; V, Cramer V on the basis of X^2 test with simulated p values; FB-food, foodborne-food, i.e. an outbreak was reported to be caused by food and the outbreak strain was detected in food; FB=feces, foodborne-feces, i.e. an outbreak was reported to be caused by food and the outbreak strain was detected in human feces; FHB, food handler-borne, i.e. an outbreak was reported to be caused by an infected food handler contaminating the food and the outbreak strain was detected in human feces; PB, person-borne, i.e. an outbreak was reported to be caused by person-to-person transmission and the outbreak strain was detected in human feces; UN, unknown, i.e. the mode of transmission was not reported or was reported to be unknown and the outbreak strain was detected in human feces.

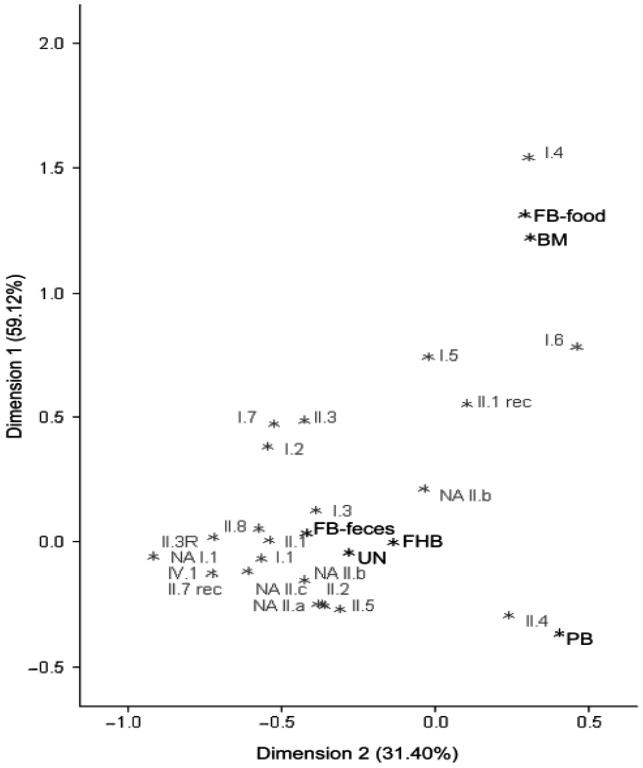

Figure 2. Two-dimensional display of the correspondence analysis of 6 norovirus genotype profiles based on nucleotide sequences in which points close to each other are similar with regard to the pattern of relative frequencies across genotypes. Dimension 1 explains 59.12% and dimension 2 an additional 31.40%. In dimension 1, foodborne-feces (FB-feces; i.e., outbreak reported to be caused by food with the outbreak strain detected in human feces only) and bivalve mollusk (BM) genotype profiles are mutually similar and differ from other profiles; the most distinct profile is person-borne (PB; i.e., an outbreak reported to be caused by person-to-person transmission with the outbreak strain detected in human feces). In dimension 2, food handler–borne (FHB; i.e., outbreak reported to be caused by an infected food handler contaminating the food with the outbreak strain detected in human feces), FB-feces, and unknown (UN; i.e., mode of transmission was not reported or was reported to be unknown with the outbreak strain detected in human feces) mutually correspond and differ from the mutually corresponding foodborne-food (FB-food; i.e., outbreak reported to be caused by food with the outbreak strain detected in food), BM, and PB.

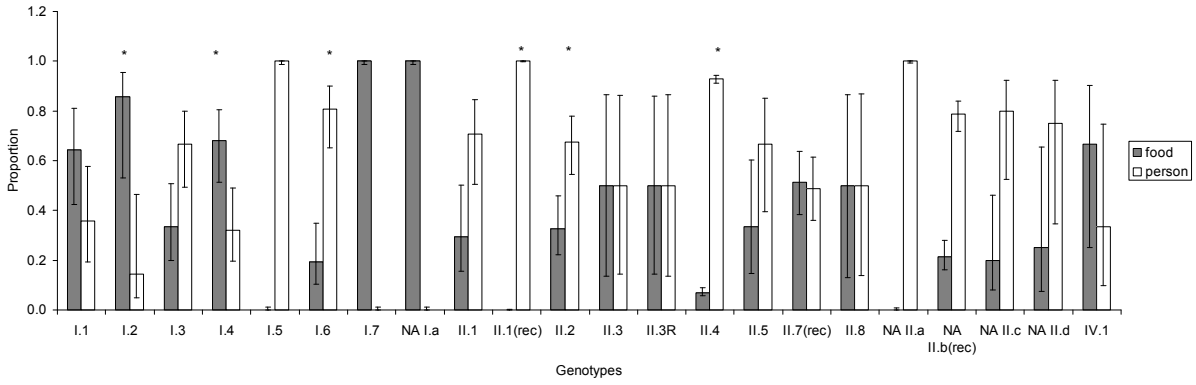

Figure 3. Estimated proportion of norovirus genotypes based on proportions as detected in foodborne outbreaks (i.e., detected in food or humans; gray bars) and person-borne outbreaks (white bars). *Indicates significance.

Discussion

Our combined epidemiologic and virologic analysis demonstrated that norovirus genotype profiles, derived from long-term norovirus strain collections, can be used to differentiate foodborne outbreaks caused by food contamination early in the food chain from those caused by food handlers contaminating food. Our study is one step in deriving practical applicable information from the existing record and possible only through the availability of continuously updated databases containing detailed epidemiologic data and virus characterization. We confirmed a significant difference in the GI:GII ratio; GI strains were more prevalent in bivalve mollusks. On the basis of the 5-year strain collections, some genotypes (I.2 and I.4) suggest FB instead of PB preference, and others (II.2 and II.6/II.7) are commonly seen in outbreaks but not detected in bivalve mollusks (and FB-food). Strains detected in food that caused outbreaks (FB-food) showed a genotype profile similar to those in bivalve mollusk monitoring and dissimilar to the profile detected in human feces (i.e., FB-feces, FHB, PB, UN) with respect to the frequently seen genotypes. This finding may reflect the ability of these genotypes to survive outside humans or their diminished ability to spread or replicate within the human population. Genotype profiles of FHB and UN resulted in diverging association outcomes, which may reflect their potential dual origin.

Although consumption of contaminated food causes both types of outbreaks, outbreaks resulting from infected food handlers clearly necessitate different measures than do outbreaks resulting from food contaminated early in the food chain. Consequently, differentiation of these modes of transmission is of interest to food safety authorities and public health institutions. Food handler–borne outbreaks are end-of-chain outbreaks easily recognized as such, as numerous outbreak reports illustrate [15, 16, 104, 115, 295]. Such outbreaks can be prevented or limited by exclusion of infected or shedding food handlers from work until 48–72 hours after recovery [15, 104, 282, 295], education of food handlers [15], and standard testing of food handlers during outbreaks [115]. A common source of contamination early in the food chain, however, may be more difficult to detect. Such contamination may result from sewage influx containing multiple viruses [60, 110, 353], making a link difficult to identify [370]. Moreover, sewage most likely contains noroviruses from person-to-person outbreaks, which can contaminate the food and thereby dilute the genotype profiling effect. Use of genotype profiles is a first step toward recognizing outbreaks resulting from contamination early in the food chain because it allows estimation of the incidence in surveillance data retrospectively and objectively minimizes misclassification of outbreaks. However, genotyping data need to be interpreted with care, and continuous updating of the database remains necessary.

Our study has some limitations. First, our measures of association could not detect differences between genotype profiles with respect to the rare genotypes. Even so, the rare outbreak or sporadic strains are of interest because they may represent potential emerging or zoonotic genotypes with consequences for public health. Types that were initially rare may remain in human surveillance, as seen with the emergence of GII.b after a large waterborne outbreak [107] followed by, among others, foodborne distribution throughout Europe. Since then, GII.b strains have caused 13% of all outbreaks (Table 1), now mainly PB, suggesting good adaptation. On the other hand, if the rare types are unable to adapt for persistence in the human population, they may be repeatedly reintroduced, causing only sporadic cases but not outbreaks. This repeated introduction of sporadic cases would remain undetected at present because routine surveillance for sporadic cases is rare [370] and is not the current

practice of FBVE. To identify the origin of newly emerging and rare strains, systematic monitoring of additional potential sources, such as cattle and swine [224] as well as sporadic human cases, is necessary.

Second, in our analysis, the transmission route was reported as unknown for 57% of outbreak strains. Incompleteness of surveillance data is a common problem [80] and has been recognized in surveillance of foodborne viral infections [246], including in the FBVE database [183, 343]. Incomplete data may have resulted in underestimation of the number of foodborne outbreaks because they may be complicated to identify. Food safety authorities routinely confirm FB clusters by detecting pathogens in food, but such confirmation is difficult for viruses because viruses, unlike bacteria, do not replicate in food, resulting in a low viral load for extraction and concentration. In addition, the matrix involved may complicate these procedures, and successful detection methods are available primarily for fresh produce with surface contamination and virus accumulating shellfish [21, 117]. However, knowledge of the prevalence of strains in the environment, foods, and humans is necessary for the interpretation of matching. Such knowledge requires monitoring, which is limited to shellfish and norovirus outbreaks [340]. For monitoring of foods other than shellfish, methods sensitive enough to detect viruses in naturally contaminated (and not spiked) food are required. The technical advisory group (TAG 4) of the Viruses in Food workgroup (WG 6) in the Technical Committee of Horizontal Methods for Food Analysis (TC 275) of the European Committee for Standardization (CEN) is validating standard methods for norovirus detection in bivalve mollusks, soft fruit, leafy vegetables, and bottled water [59]. Until such methods are available and provide knowledge about the prevalence of viral presence in foods, the use of genetic profiles retrospectively derived from outbreak surveillance data is likely to improve foodborne viral surveillance. Because the norovirus strain population is continuously evolving, our analysis needs to be repeated periodically to ensure that retrospective findings remain predictive.

Third, international comparison of norovirus strains is complicated because of their genetic diversity and the involvement of several laboratories in diagnosis; consequential different assays result in sequences with diverging lengths and from diverging genomic regions. However, this limitation is not likely to have influenced our results because it affects mostly the comparison of sequence clusters and not genotypes. Moreover, within FBVE, standardization of diagnostic methods occurs by having participating laboratories regularly test a representative panel of fecal samples [348].

We showed that norovirus genotype profiles can be used to estimate the foodborne proportion of norovirus outbreaks while excluding those of the food handler as a source. Distinction at genogroup level had already indicated epidemiologic differences [343], and we have now demonstrated that genotype profiles can be used to differentiate transmission modes. The profiles and proportions are likely to be helpful for estimating the number of outbreaks with potential of causing geographically disseminated outbreaks. Because identification and investigation of such outbreaks provides insight into effective prevention measures during the production process, detection should enable containment of viral foodborne infection and thus prevent further spread and the consequent potential for large numbers of human infections.

An Integrated Approach to Identifying International Foodborne Norovirus Outbreaks

Chapter 7

Linda Verhoef, Roger D. Kouyos, Harry Vennema, Annelies Kroneman, Joukje Siebenga, Wilfrid van Pelt, Marion Koopmans, on behalf of the Foodborne Viruses in Europe network.

Emerg Infect Dis 2011[e-pub ahead of print]

Abstract

International foodborne norovirus outbreaks can be difficult to recognize when using standard outbreak investigation methods. In a novel approach, we provide step-wise selection criteria to identify clusters of outbreaks that may involve an internationally distributed common foodborne source. After computerized linking of epidemiologic data to aligned sequences, we retrospectively identified 100 individually reported outbreaks that potentially represented 14 international common source events in Europe during 1999–2008. Analysis of capsid sequences of outbreak strains (n = 1,456), showed that 7% of outbreaks reported to the Foodborne Viruses in Europe database were part of an international event (range 2%–9%), compared to 0.4% identified through standard epidemiologic investigations. Our findings point to a critical gap in surveillance and suggest that international collaboration could have increased the number of recognized international foodborne outbreaks. Real-time exchange of combined epidemiologic and molecular data is needed to validate our findings through timely trace-backs of clustered outbreaks.

Introduction

Noroviruses are the most prevalent causative agents of acute viral gastroenteritis in the community [73, 147, 336, 355]. Currently, 5 norovirus genogroups have been described, subdivided into at least 40 genotypes [120, 174], but most clinical impact in recent years is caused by viruses from a single genotype in genogroup II, the GII.4 [1, 182, 205, 341]. The symptoms of norovirus disease are usually mild and self-limiting, but there is some evidence that the disease can contribute to mortality [20, 281]. Infection occurs via the gastro-intestinal tract after contact with infected persons, after ingestion of contaminated food or aerosols, or through environmental contamination [18, 86].

As the various modes of transmission call for quite distinct control measures, it is important to assess which proportion of disease can be attributed to which mode of transmission. However, this question is difficult to answer. Due to the high rate and rapidity of secondary spread of norovirus infection following a foodborne introduction, outbreaks initially linked to a food-source may present as person-to-person outbreaks by the time they are recognized. Even if food is suspected, confirmation of the source is complicated. Virus detection in food commodities is possible but hampered by such factors as low levels of norovirus in food, food matrix complexity, genetic variability of norovirus [192], the absence of an efficient cell culture system to propagate human noroviruses [85], and the fact that leftovers are often not available for pathogen detection.

Given the globalization of the food-market, diffuse international outbreaks are likely to occur [155, 290]. For public health officials, these may present as outbreaks that appear to be “regular” person-to-person outbreaks, because infection of one or a few persons with viruses through food consumption will not be noticed unless it triggers an outbreak due to secondary spread, or if the food was consumed by multiple persons which may trigger an outbreak investigation directed at identifying a food source. However, the identification of international links is complicated. Viruses remain infectious in frozen ready-to-eat products over prolonged periods, and linked outbreaks are likely to be separated in time [93]. Other problems are the mutation rate of viruses resulting in non-identical strains from a common source [248], sewage contamination with multiple non-similar strains during production of

shellfish or crops [25], underreporting of cases [246, 247], and incompleteness of outbreak reports [183, 343], as well as the unknown background level of viruses in foods, the environment, or asymptomatic shedders. Clearly, methods combining molecular and basic epidemiological criteria are needed to assist public health efforts to identify international foodborne outbreaks.

For this reason we performed a retrospective analysis of norovirus outbreak surveillance data collected since 1999 by Foodborne Viruses in Europe (FBVE), a combined laboratory and epidemiology network [174]. Strain sequences from outbreaks linked to a common source are expected to be more similar than strains from outbreaks with a different source [212]. We sought to quantify strain variability within and among molecular sequence clusters of multiple outbreaks and the consequences of using various similarity cut-off values to identify outbreaks with probable links to other outbreaks. By this approach we aimed to retrospectively identify potential common-source events that were not detected by routine investigations and also to provide criteria that may assist in detection of such events.

Methods

Definitions

A **norovirus outbreak** was reported to FBVE when it included a minimum of 2 patients in the same area within 2 days showing ≥2 times of vomiting and/or watery diarrhea within 24 hours [159, 174]. A **gastroenteritis outbreak** was ascribed to norovirus based on compatible descriptive epidemiology and laboratory confirmation in at least 2 of 5 feces samples tested [87]. An **outbreak strain** is a sequenced norovirus strain considered representative of an outbreak (found preferably in at least 2 samples from patients in the same outbreak). If dissimilar sequences were detected, multiple strains were considered representative. A **genotype** is a group of closely related noroviruses, i.e., showing >80% similarity in the complete capsid amino acid sequence. Genotypes can be assigned based on shorter sequences if a full capsid was previously identified and sequenced for comparison [119]. **A cluster** refers to a molecular cluster of multiple outbreaks (i.e. *not* an epidemiological cluster of patients in 1 outbreak). **A cluster of similar sequences** is a group of outbreak strain sequences in the same genotype that show a minimal number of mutations within the region of overlap; the exact number of mutations depends on the sequence length in the region and the cut-off value used to define similarity. **A cluster of identical sequences** is a group of outbreak strain sequences with the highest possible similarity (100%). According to reporting standards of the FBVE network [183, 209], the suspected mode of transmission during an outbreak was considered **foodborne (FB)** when the infection was related to consumption of food contaminated during its production or processing; **foodhandler-borne (FHB)** when infection related to food prepared by an infected food-handler; **person-borne (PB)** when it related to direct contact with infected persons; and **unknown (UN)** when no mode could be identified.

Selection of Strains Representing Outbreaks

Between January 1999 and November 2008, the FBVE network collected molecular information on a total of 5499 norovirus outbreaks in Denmark, Finland, France, England and Wales, Germany, Hungary, Ireland, Italy, the Netherlands, Norway, Slovenia, Spain and Sweden [183, 209]. Strengths and limitations of the FBVE data collection have been described [183]. Although the name FBVE suggests a foodborne focus, the network actually

investigates outbreaks of viral gastroenteritis with all modes of transmission. It seeks to obtain a comprehensive overview of viral activity in the community and to allow capture of foodborne outbreaks that have evaded recognition. FBVE data is reported aggregated at outbreak level. Consequently, throughout the analysis here described:

- a strain represents an **outbreak** (i.e. outbreak-representative strain)
- a cluster is a **molecular cluster of outbreaks** (i.e. cluster of outbreak-representative strains)

As the norovirus genome shows its highest variability in the capsid, comparing sequences from this region will yield the lowest number of identical strains [363]. Therefore, regions C and D, both located in ORF2 at the capsid gene, were our regions of choice for identification of linked outbreaks. All norovirus outbreak strains reported to the FBVE network between 1999 and 2008 were included if a full or partial capsid sequence was involved. This yielded 1504 outbreak-representative strain sequences reported by all above-mentioned countries. Sequence lengths varied between 90 and 1640 nucleotides. We used sequences including ORF2 nucleotide positions 1-300 (93%) and other targets within ORF2 nucleotide positions 300-1620 (7%), including full capsid genes (8%).

Assignment of Genotypes

Using the typing tool (www.rivm.nl/mpf/norovirus/typingtool) we classified genotypes on the basis of their similarity to reference strains representing known genotypes. In this paper, the ORF2 reference set was used for genotyping.

Alignments of Strains Representing Outbreaks

Nucleotide sequence alignment and similarity calculation according to the neighbour-joining (NJ) method was performed for all ORF2 outbreak strain sequences within genotypes by using Bionumerics 5.1 (Applied Maths, Belgium). If sequences from non-overlapping nucleotide positions were included, these sequences were separately aligned within the involved nucleotide positions.

Analysis of Clustering Strains Representing Outbreaks

Alignments were imported into the R project version 2.8.0 (http://cran.r-project.org) for analysis in 6 steps (Figure 1). In step 1, the APE [255] and the seqinR [49] packages in R were used to assign numbers to clusters of identical outbreak strain sequences, according to pair-wise comparison of strains within genotypes. Cluster numbers were assigned to enable rapid and computerized linking of the large molecular and epidemiological data sets and allow systematic statistical analysis of combined data.

In step 2, the characteristics of outbreak strain sequence clusters were compared with respect to the following aspects: frequency of clusters within genotypes, sizes of clusters, the overlapping number of nucleotides, number of countries involved, time-period over which outbreaks were reported, transmission modes as reported in the categories FB, FHB, PB and UN. Based on available information, reported sources of infection in FB outbreaks were allocated to the following categories: filter-feeding bivalve **shellfish** (including oysters and mussels); **berries** (including raspberries, blueberries and strawberries); **water** (including water related to food preparation, irrigation, and contaminating floods, but no shellfish or berries reported); **RTE** (including ready-to-eat foods like bread, sandwiches, layer cakes, delicatessen, salad, but no shellfish or berries reported

as one of the ingredients) and **other** (including self-served meals, buffet or catering, with multiple food items but none of the previous food classes reported). Outbreak strain sequence clusters that included at least one foodborne outbreak were selected for further analysis, and designated 'possible FB clusters'.

In step 3, using the APE package of R, all 'possible FB clusters' were stepwise extended to include sequences with similarity of 99.5%, 99%, 98%, 97%, 96% and 95%. This was done to determine the cut-off level, i.e. the level of similarity, needed to recognize potentially linked outbreaks and thereby to assist in the confirmation of definitely linked outbreaks.

In step 4, p-values were calculated to determine association with food for a chosen cut-off level. To do so, the frequencies of the transmission mode for each strain were considered a random draw from the frequencies of this transmission mode in the background population in the database as a null hypothesis, i.e. as random draws from a binomial distribution and thus the probability of finding such a cluster by chance. For example, a cluster of 5 strains including 2 FB (i.e. 40%) has a probability of 0.02 to be found coincidentally in a total dataset containing 5% FB outbreaks. This cluster is then considered to be significantly associated with foodborne transmission. Such calculations were done to determine i) the association of genotypes with foodborne transmission, i.e. 'FB genotype', with the frequency of foodborne outbreaks in the genotype considered as a random draw from the total dataset; ii) association of clusters with foodborne transmission, i.e. the frequency of the foodborne mode of transmission for the specific cluster considered as a random draw from all strains in the genotype; and iii) association of clusters with a specific food class, i.e. the frequency of the food class for the specific cluster considered as a random sample from all foodborne outbreaks. These calculations were the basis for the transition from 'possible' to 'probable FB clusters'.

In step 5, the calculations of step 4 were used to cut down all 'possible FB outbreaks to 'probable FB outbreaks', i.e. those clusters which were more likely to be related to food. The clusters significantly and borderline significantly associated with food were selected according to 3 selection criteria: i) all 'possible FB clusters' in 'FB genotypes'; and for the 'non-FB-genotypes': ii) those 'possible FB clusters' that were significantly or borderline significantly associated with FB transmission; and iii) those 'possible FB clusters' that were significantly or borderline significantly associated with a specific food class. Clusters selected through these criteria were designated 'probable FB clusters'. P-values were considered significant if $p < 0.05$ and borderline significant if $0.05 < p < 0.10$.

In step 6, outbreaks that could be linked and internationally disseminated were selected from 'probable FB clusters' if they involved at least 2 countries.

Verification of Outcomes

The above selection criteria were applied to the FBVE database to retrospectively identify clusters of outbreaks that may have involved international dissemination of food. To verify the criteria of the approach as described above, the selected clusters were compared with the clusters previously reported to FBVE as linked outbreaks, as a measure of sensitivity of the approach (i.e. ability to detect true clusters).

Table 1. Aggregated data of clustered outbreak strains sequences and properties of the outbreaks within such clusters for each of the genotypes (GT)

GT	Total no. strains	No. (%) strains in clusters	No. (%) strains not in clusters	Minimum similarity within GT in %	Total no. Strain clusters	size strain clusters: range*	size strain clusters: median	No. (%) FB outbreak strains	No. sign possible FB clusters	No. (%) FHB outbreak strains	No. sign FHB clusters	No. (%) PB outbreak strains	No. sign PB clusters	No. (%) UN outbreak strains	No. sign UN clusters
I.1	24	19 (79)	5 (21)	91.2	4	2-7*	5	8 (33)†	1§	0 (0)	0	2 (8)	0	14 (58)	0
I.2	34	20 (59)	14 (41)	97.5	5	2-6	5	5 (15)	1§	0 (0)	0	0 (0)	0	29 (85)‡	0
I.3	43	16 (37)	27 (63)	78.3	5	2-6	4	6 (14)	1§	0 (0)	0	2 (5)	0	35 (81)	0
I.4	40	23 (58)	17 (42)	94.8	8	2-5	2	8 (20)†	1§	2 (5) †	1§	0 (0)	0	30 (75)	0
I.5	6	5 (83)	1 (17)	93.1	2	2-3	2 .5	2 (33)‡	0	0 (0)	0	0 (0)	0	4 (67)	0
I.6	20	13 (65)	7 (35)	88.7	4	2-5	3	4 (20)‡	1**	1 (5)‡	0	2 (10)	0	13 (65)	0
II.1††	28	10 (36)	18 (64)	90.9	4	2-3	2.5	6 (21)†	0	0 (0)	0	12 (43)†	0	10 (36)	0
II.2	30	9 (29)	21 (71)	82.4	4	2-3	2	11 (37)†	0	0 (0)	0	3 (10)	0	16 (53)	0
II.3††	136	70 (52)	66 (48)	90.5	14	2-15	3	15 (11)	0	0 (0)	0	35 (26)†	4§**	86 (63)	0
II.4††	950	691 (73)	254 (27)	82.4	47	2-361	3	41 (4)	3§**	2 (0)	0	178 (19)‡	3§**	729 (77)†	4§**
II.5	3	2 (66)	1 (33)	97.0	1	2	2	1 (33)	0	0 (0)	0	0 (0)	0	2 (67)	0
II.6††	54	30 (56)	24 (44)	92.0	8	2-15	2	11 (20)†	1§	1 (2)	0	6 (11)	1§	36 (67)	0
II.7	57	39 (68)	18 (32)	96.2	4	2-28	3	13 (23)†	1**	0 (0)	0	6 (11)	1§	38 (67)	0
II.8	4	2 (50)	2 (50)	96.0	1	2	2	2 (50)†	0	0 (0)	0	0 (0)	0	2 (50)	0
II.10	3	0 (0)	3 (100)	97.0	0	NA	NA	0 (0)	0	0 (0)	0	2 (67)‡	0	1 (33)	0
II.11	1	0 (0)	1 (100)	NA	0	NA	NA	0 (0)	0	0 (0)	0	0 (0)	0	1 (100)	0
II.12	4	0 (0)	4 (100)	93.9	0	NA	NA	0 (0)	0	0 (0)	0	0 (0)	0	4 (100)	0
II.13	3	0 (0)	3 (100)	96.6	0	NA	NA	1 (33)	0	0 (0)	0	0 (0)	0	2 (67)	0
II.14	3	0 (0)	3 (100)	99.5	0	NA	NA	1 (33)	0	0 (0)	0	0 (0)	0	2 (67)	0
II.16	4	0 (0)	4 (100)	97.5	0	NA	NA	0 (0)	0	0 (0)	0	1 (25)	0	3 (75)	0
II.17	1	0 (0)	1 (100)	NA	0	NA	NA	0 (0)	0	0 (0)	0	0 (0)	0	1 (100)	0
II.NA1	6	2 (50)	4 (50)	94.2	1	2	2	0 (0)	0	0 (0)	0	2 (50)	0	2 (50)	0
II.NA3	1	0 (0)	1 (100)	NA	0	NA	NA	0 (0)	0	0 (0)	0	0 (0)	0	1 (100)	0
Total	1456	938 (64)	518 (36)		112	2-364		136 (9)	10	6 (0)	1	252 (17)	9	1062 (73)	4

*Size of a strain cluster is the number of outbreak-representative strains within a molecular cluster of outbreaks, i.e. 2-7 outbreaks (not 2-7 patients per outbreak);† Genotypes with significantly higher proportion ($p<0.05$) of outbreaks with this transmission mode, when compared to the proportion in the total sequence population; ‡ Genotypes with borderline significantly higher proportion ($0.05<p<0.10$) of outbreaks with this transmission mode, when compared to the proportion in the total sequence population; § Clusters with significantly higher proportion ($p<0.05$) of outbreaks with this transmission mode, when compared to the proportion within the genotype; ** Clusters with borderline significantly higher proportion ($0.05<p<0.10$) of outbreaks with this transmission mode, when compared to the proportion within the genotype; †† Genotypes II.1, II.3, II.4 and II.6 were aligned separately for different genomic regions.

Estimate of Proportion of Common-source Foodborne Outbreaks

The frequency of linked foodborne outbreaks, both at national and international level, was calculated as a proportion of the total number of reported outbreaks, based on the above analyses. Due to uncertainty of the causal, consequential or coincidental relationship between outbreaks in molecular clusters, estimates were given for low, most likely and high values of the number of linked foodborne outbreaks. Low values were calculated as the actual frequency of outbreaks in the specific clusters reported to be foodborne. High numbers were the total number of outbreaks in the specific clusters, i.e. including all reported transmission modes. For the likely values, the outbreaks from unknown transmission were extrapolated proportionally to the FB outbreaks reported in the specific cluster. Thus, likely values were calculated as follows: for a cluster of *x* outbreaks containing *a* FB, *b* PTP and *c* FHB and *d* UN outbreaks, for high value is *x*, the low value is *a*, and the likely value is $a + \frac{a}{a+b+c} * d$. Likely values with range for low and high values were subsequently compared with results of the epidemiological overviews currently used in outbreak reporting in Europe [183, 343].

Results

Assignment of Genotypes

Genotyping resulted in clustering of reported outbreak strains into 23 ORF2 genotypes for 1456/1504 (97%) sequences. For the remaining 48 (3%), sequence data provided were insufficient for assignment of a genotype.

Cluster Analysis

The degree of strain similarity and the proportion of clustering strains (Figure 1, steps 1 and 2) varied greatly among genotypes (Table 1). A total of 112 clusters of identical (100% similarity) outbreak strains were found, with 938/1456 (64%) of all reported outbreaks found in clusters. Of these, 38 sequence clusters involving 654/938 (70%) outbreaks included at least one FB outbreak. These were designated ‘possible FB clusters’, i.e. possibly representing linked foodborne outbreaks (Figure 1, step 2). When the cut-off for strain similarity was lowered step-wise in R (Figure 1, step 3), logically, the number of distinctive clusters decreased, whereas the size of each cluster increased. The similarity cut-off differed between genotypes. Six genotypes (I.1, I.4, I.5, II.1, II.5 and II.8) yielded a cluster of strains that remained distinct regardless of the cut-off used. For the other genotypes, lowering the cut-off to similarity levels of 99.5% or 99% showed a sharp drop in the number of distinct clusters, i.e. less clusters that as a consequence increased in size. For 7/14 genotypes the number of such clusters dropped to 50% at cut-off value 99.5%. At 99%, this was the case for 10/14 genotypes (data not shown). As we aimed to provide a conservative estimate for linked outbreaks for all genotypes, 100% similarity was chosen as the cut-off for further analysis steps.

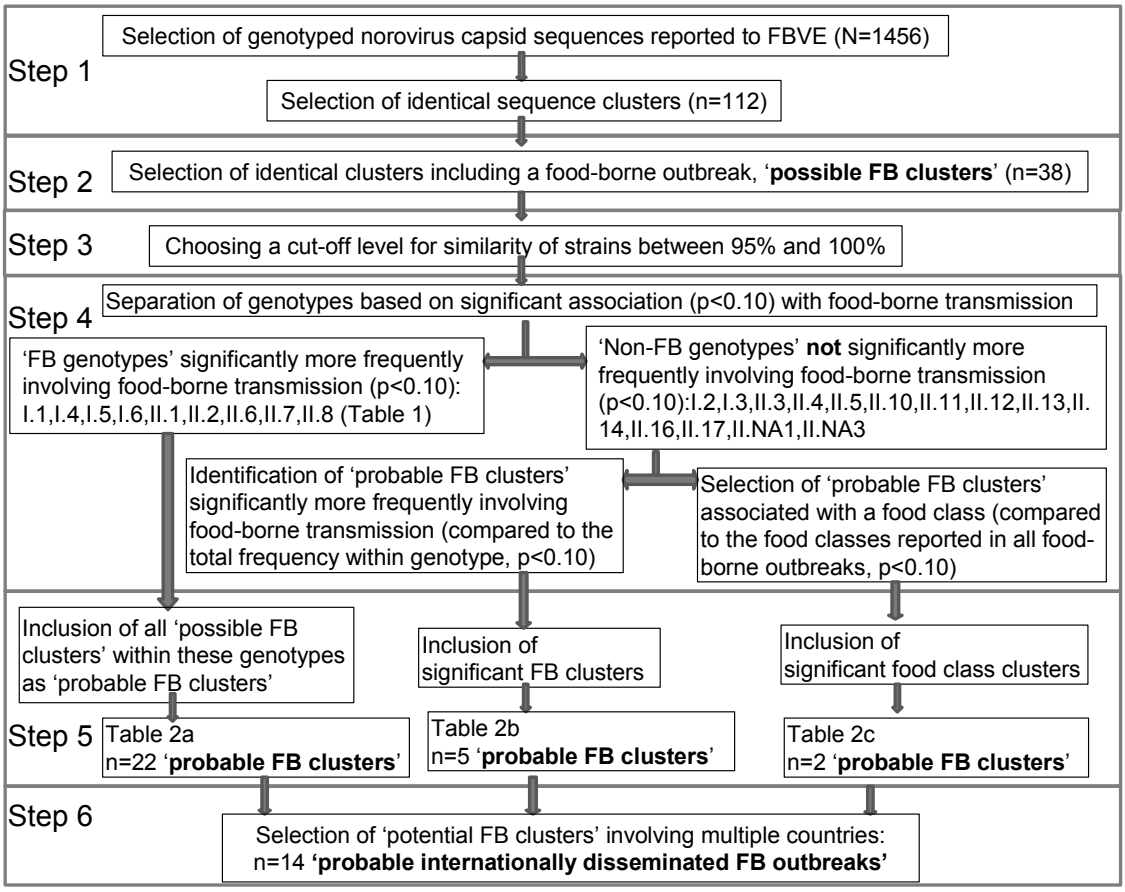

Figure 1. Selection of foodborne clusters of strains potentially representing internationally disseminated common-source outbreaks. Selection involved 6 steps, according to combined epidemiological and molecular criteria. Each analyzed strain represented an outbreak.

Probable foodborne clusters' of outbreaks were selected from 38 'possible foodborne clusters' based on 3 criteria for statistical association with food (Figure 1, step 4): 1) Table 2a shows 22 clusters in nine genotypes (I.1, I.4, I.5, I.6, II.1, II.2, II.6, II.7, II.8) significantly or borderline significantly more often contained FB outbreaks, as compared to the total dataset; 2) Table 2b shows 5 additional clusters in 'non-FB-genotypes' for which the specific transmission mode FB was more frequently reported than in the genotype. 3) 2 additional clusters were significantly associated with a food class, when compared to the frequency of these food classes reported for all FB outbreaks (Table 2c). Fourteen of these 29 'probable FB clusters' (48%) involved more than one country and are therefore labeled as 'probable internationally disseminated foodborne outbreaks' (Figure 1, step 6).

Validation of Criteria

In the FBVE data set of 1456 capsid sequences, 30 outbreaks had previously been identified as linked outbreaks in 10 clusters, based on standard epidemiological investigation [183]. In contrast, in the present study 29 clusters of interest involving 122 likely linked outbreaks (range low-high: 51-166) were retrospectively identified in this study (Table 2; newly identified clusters indicated in bold). Of the 10 previously reported FBVE outbreak clusters, 8 (80%) were identified using the approach described in this paper. These 8 clusters involved 32 likely linked outbreaks (range low-high: 18-69) and included 2 international and 3 national

clusters, plus 3 clusters reported as national but containing sequences identical to those from outbreak strains reported elsewhere. The 2 FBVE clusters that were missed by our analysis involved 1) 3 outbreaks with 3 different genotypes involved, and 2) 3 outbreaks for which 2 different food classes were reported (RTE and 'other'). Both food classes ended up non-significant for this cluster in step 5 (Figure 1) of the analysis.

International Clusters Potentially Linked through a Common Source

Previously, 36 of 1456 (2.5%) outbreaks reported through the FBVE network had been linked to a common source, of which 6 (0.4%) involved events in multiple countries. Using the stepwise criteria described here resulted in a significant increase to 122 of 1456 (8.4%, range low-high: 51-166) potential common-source outbreaks, of which 97 (6.7%, range low-high: 29-130) involved events in more than one country (data not shown).

Discussion

Our analysis suggests that 7% (range low-high 2-9%) of norovirus outbreaks reported through the FBVE network are likely to be international outbreaks with a common source. Our estimate is at least 5-fold higher than the 0.4% recognized through routine investigations. We showed that the proportion of linked foodborne outbreaks can be estimated with a sensitivity of 80%, using step-wise selection criteria combining molecular and epidemiological information and derived from a large background dataset. The computerized linking of epidemiological data to aligned sequences in R project for statistical computing considerably reduced the time needed for analysis and was an essential prerequisite of this novel approach. As sequencing becomes less expensive and public health data bases expand, the utility of our approach for public health decision-making will increase in the future [140].

Several research groups have made efforts to estimate the burden of norovirus and foodborne disease, finding that viral illness varies between 1/780 UK inhabitants and 1/33 US inhabitants [9, 73, 126, 229]. For Europe, we previously estimated that 21% of all norovirus outbreaks were caused by food [345], but that report did not consider potential (international) links between outbreaks. In our current study, we found that 2-9% of all reported outbreaks may be linked to a common source with international distribution. Since this study was done retrospectively, we could not collect additional data to verify suspected clusters. To prove this with certainty, the analysis should be done in real-time, and involve more in-depth outbreak investigations to establish a risk food with epidemiological approaches and possibly food testing. However, we do see this as a novel approach to provide the basis for estimates of the burden of foodborne disease. Studies done so far, have used gross extrapolations of data estimating the proportion of reported noroviral disease that can be attributed to food, but have not included the burden of outbreaks. To our knowledge, this study is the first attempt at providing a basis for such estimates, and especially the proportion attributable to foodborne transmission.

A shortcoming of our method is that the sequences used are mostly originating region C of the capsid genome, which is known as a conserved region [225]. Thus, clusters may become smaller when based on the more variable regions like the P2 domain [364]. On the other hand, our approach is likely to provide a conservative estimate, as it relies on identical sequence clusters and does not include outbreaks caused by strains that are

Table 2. Characteristics of 29 strain sequence clusters with identical capsid sequences that may share a common exposure through food. Outbreak clusters were selected based on one of 3 criteria: 2a) genotype preferentially found in foodborne outbreaks; 2b) foodborne mode of transmission more commonly reported for cluster and 2c) specific food item more commonly reported in given cluster of outbreaks.

Cluster	#strains (% of total in GT)	Minimum # NT overlap	Transmission mode based on reports* (p)	Source category	Time span	Countries involved	Reported to FBVE as linked outbreak
Table 2a	Selection criterion 1: identical clusters within 'FB genotypes' including at least 1 FB outbreak, n=22						
1 (I.1)	7 (29)	204	UN? (0.38)	Shellfish	Sep-Dec 2004 and Feb 2006	FR, HU, GB	Yes, in FR
2 (I.1)	4 (17)	204	FB (0.01)	Shellfish	Apr-Nov 2000 and 2001	FR, NL	Yes, in FR
3 (I.1)	2 (8)	206	FB? (0.11)	Unknown	2000	FR	No
4 (I.1)[†]	**6 (25)**	**221**	**UN? (0.20)**	**Unknown**	**Dec 2000 – Dec 2005, 2006**	**FR, GB, SE**	**No**
5 (I.4)	**5 (13)**	**204**	**FB? (0.45)**	**Water, berries**	**2000, Feb 2002 – Aug 2006**	**NL, FR, SE**	**No**
6 (I.4)	2 (5)	204	FB (0.04)	Shellfish	2000, 2001	NL	No
7 (I.4)	2 (5)	215	FHB (0.00)	RTE	Mar 2006	DK	Yes
8 (I.5)	**3 (50)**	**204**	**FB? (0.25)**	**Shellfish, delicatessen**	**Nov 2004 – Feb 2006**	**IE, FR**	**No**
9 (I.6)	**5 (25)**	**204**	**FB (0.07)**	**Self-served meal, shellfish**	**Dec 2002 – Jul 2006**	**HU, NL, SE**	**No**
10 (I.6)	3 (13)	291	FHB? (0.14)	Self-served meal	Sep 2002 – and 2004	SE	No
11 (II.1)	3 (11)	272	FB? (0.11)	Water	Sep 2000 – Jan 2001	FR	No
12 (II.1)	3 (11)	268	FB?(0.29)	Unknown	Jan-Mar 2001	NL	No
13 (II.2)	2 (6)	255	FB? (0.14)	Shellfish	Dec 2006 and Mar 2007	FR	No
14 (II.2)	**2 (6)**	**235**	**FB? (0.60)**	**Shellfish**	**Jan 2005 and Feb 2008**	**FR, GB**	**No**
15 (II.2)	2 (6)	338	FB? (0.60)	Water	Oct 2002 and Mar 2003	SE	No
16 (II.6)	2 (4)	255	FB (0.04)	Paella	Sep 2007	FR	Yes

17 (II.6)	15 (28)	45	PTP? (0.22)	Berries, RTE	Jan 2004 – Dec 2006	HU, DK, GB, FR, NL, SE, IT	Yes, multiple countries and genotypes
18 (II.6)	**2 (4)**	**196**	**FB? (0.36)**	**Self-served meal**	**Jan – May 2006**	**GB, NL**	**No**
19 (II.7)	**28 (49)**	**94**	**UN?(0.55)**	**Self-served meal, RTE**	**Jun 2003 – Apr 2006**	**GB, DK, NL, FR, SE**	**No**
20 (II.7)	2 (4)	337	FB (0.05)	RTE	Feb 2004	SE	No
21 (II.7)	5 (9)	249	PTP (0.01)	RTE	Dec 2005 – Mar 2006	SE	No
22 (II.8)	2 (50)	338	FB? (0.25)	Berries	Jun and Aug 2006	SE, PL	Yes, multiple genotypes, link abroad
Table 2b		Selection criterion 2: identical clusters significantly associated with foodborne transmission, n=5					
23 (I.2)	2 (6)	206	FB (0.02)	Unknown	Apr 2000	FR	No
24 (I.3)	2 (5)	206	FB (0.02)	Shellfish	Apr 2000	FR	No
25 (II.4)	35 (4)	140	FB (0.05)	RTE, Shellfish, berries	Jan 2006 – Nov 2006	FR, DK, SL, IE, HU, SE, IT, NL	Yes, local link hospitals
26 (II.4)	2 (0)	238	FB (0.08)	Unknown	Feb 2008	FR	Yes
27 (II.4)	2 (0)	249	FB (0.08)	Unknown	Nov 2000 and dec2001	NL	No
Table 2c		Selection criterion 3: identical clusters statistically associated with a specific food class (shellfish, berries, water, other), n=2					
28 (I.3)	**4 (9)**	**234**	**FB? (0.45)**	**Water, berries**	**2000, Feb 2002 – Aug 2006**	**FR, DE, SE**	**No**
29 (II.4)	**12 (1)**	**227**	**UN? (0.20)**	**Shellfish**	**Oct 2007 – Nov 2009**	**FR, IE**	**No**

* frequency of transmission modes as reported for the single outbreaks within the specific cluster were considered a random draw from the frequencies of this transmission mode from the background population in the Foodborne Viruses in Europe database, i.e. as random draws from a binomial distribution. P-values <0.10 were considered significant or borderline significant. P-values >0.10 were considered non-significant. However, for clusters with none of the transmission modes ending up with a significant p-value, the transmission mode with the smallest p-value was chosen and presented with a question mark (?);† Outbreak clusters in bold were international outbreaks newly identified through the analysis in this chapter; other international outbreak clusters had already been reported as (suspected) common source events.

phylogenetically closely related. Given the mutation rate of genotype II.4 noroviruses [304], closely related strains could well represent linked outbreaks. This mutation rate, as well as the similarity cut-off, may be genogroup or genotype specific (Table 1). When a single similarity cut-off for all genotypes is used as a selection criterium, any mutation counts equally. Nevertheless, a particular mutation may indicate that strains share a common ancestor, and a mutation in a particular genotype may indicate either a longer or shorter genetic distance. Therefore, phylogenetic analysis is needed to identify additional linked outbreaks involving closely related strains.

Another shortcoming of our methods is that we would miss common source events that involve more than one strain, as has been described in some examples that involved sewage-contaminated shellfish [93, 107, 174]. Nevertheless, we detected 3 out of 4 linked outbreaks involving multiple genotypes, indicating that such outbreaks are likely to show other characteristics that can be captured by our criteria.

A prerequisite for our approach is the availability of combined epidemiological and laboratory data. National surveillance systems differ in their potential for matching these data, in the intensity of surveillance, and in the attention given to foodborne outbreaks. With no special focus, foodborne outbreaks are likely to be underreported, as recognition will be complicated by the rapidly taking over person-to-person transmission [341]. Moreover, many persons are involved in data-entry, which may have had consequences for data-quality [183]. This may hamper international comparison of data to detect foodborne outbreaks [343]. For instance, France and Denmark have been reporting nearly only foodborne outbreaks, whereas other countries include a wider range of transmission modes. An added value of our approach is reflected in its finding of foodborne outbreaks in France and Denmark and likewise in other countries, like the United Kingdom and Germany, that are less focused on identification of foodborne outbreaks. Despite the fact that underreporting of foodborne outbreaks in our dataset was likely, our criteria may thus provide insight into the number of foodborne outbreaks occurring in countries whose surveillance systems may miss such outbreaks.

In conclusion, combined epidemiological and molecular analysis can recognize internationally disseminated outbreaks that may share a common foodborne source. Step-wise selection criteria can be derived from an extensive background data set and used to retrospectively estimate the proportion of international outbreaks that share a foodborne source. Prospective use of the criteria needs to be validated through real-time data sharing and timely follow-up of outbreak clusters. Our findings nevertheless show that current surveillance has a critical gap, which can be bridged through systematic analysis of combined molecular and epidemiological data.

Data-driven Selection of the Informative Genomic Region for Linking Outbreaks: Norovirus as an Example

Chapter 8

Linda Verhoef, Kelly Williams, Annelies Kroneman, Bruno Sobral, Harry Vennema, Wilfrid van Pelt, Marion Koopmans, on behalf the FBVE network.

Submitted for publication.

Abstract

The recognition of common source outbreaks is supported by finding identical sequences in patients. Pathogen sequencing has been established in many (national) public health laboratories and academic centers, but often partial and different genome sequences are used. Therefore, agreement about the target sequences that have sufficient diversity to use them to resolve links between outbreaks is crucial. Although harmonization of laboratory methods is one of the keystone activities of networks that have the aim to identify common source outbreaks, this has proven difficult to accomplish, particularly in the international context. Here, we aimed to provide a generic method for obtaining the informative genomic region enabling identification of common source – international – outbreaks by using a bio-informatics analysis of currently available data. Given a rich dataset of combined epidemiological and virological data available for noroviruses, this pathogen was used as an example. We analyzed 502 unique full capsid gene sequences available from the public domain, including capsid sequences known to represent linked cases. We evaluated over 3000 so-called simulated PCR products (sequences of different lengths and targets) for robustness and specificity of clustering of known linked outbreaks against the background diversity of strains. Great differences were seen in the robustness of commonly used PCR targets for cluster detection. Reliability of this approach depends on the quality of the background dataset, and we recommend periodic reassessment of this growing dataset to verify the conclusions. The approach should be applicable to multiple sequence-based datasets of other pathogens as well.

Introduction

Cluster detection based on identification of related pathogen sequences in patients is an important tool to support outbreak investigations in modern day public health and clinical laboratories. The added value of these approaches is particularly clear in detection of links that are difficult to unravel through classical epidemiological investigations, for instance in diffuse foodborne outbreaks involving several countries. Viral contamination of food can occur during production, through infected food handlers, or the use of sewage contaminated water during cultivation, production and processing of foods. Unfortunately, the standard legally-required quality-control criteria for food are not adequate for detection or exclusion of viral contamination [103, 319]. Given the globalization of the food market, a single batch of food is often consumed in several countries simultaneously [155], and may consequently cause international viral outbreaks. A foodborne viral source can be identified by comparing viral ‘fingerprints’, i.e. a nucleotide (nt) sequence, which would enable measures like withdrawing the product from the market. However, such decisions depend on the timely detection of viruses in food and patients, and correct interpretation of sequence comparison between countries. A prerequisite for comparing sequences internationally is the presence of a large enough up-to-date background dataset representing viral presence in the community, which is collected according to standardized detection and typing methods. Such data provide knowledge on the background diversity of strains, but may only be available for a select number of countries [259, 260].

The need for standardized molecular surveillance is a general problem increasingly recognized for food- and waterborne disease. Several electronic surveillance systems for norovirus outbreaks have recently been initiated in the US (Calicinet), Canada (Vironet), Australia and New Zealand (Norovirus Surveillance Network) and globally (Noronet) [308]. The Foodborne Viruses in Europe (FBVE) network was one of the pioneers collecting both laboratory and epidemiological data on norovirus outbreaks in Europe since 1999 [174]. Noroviruses (NoVs) are among the most prevalent causative agents of community acquired viral gastroenteritis [73, 355]. NoVs are a genetically diverse, single-stranded, positive-sense RNA virus group within the *Caliciviridae*, consisting of a ~7.5-kb genome in three open reading frames (ORFs). The first ORF (ORF1) encodes a polypeptide whose processing yields multiple non-structural proteins; ORF2 encodes the viral capsid protein (VP1); and ORF3 encodes a minor structural protein (VP2). Currently five norovirus genogroups have been described and subdivided into at least 40 genotypes based on their amino acid capsid sequence [120, 174, 186]. Most impact in recent years is caused by viruses from a single genotype in genogroup II, the GII.4, causing illness worldwide [304, 308]. The symptoms of NoV illness are usually mild, but there is some evidence that the disease can contribute to mortality among the elderly [20, 281]. Infection occurs via the gastro-intestinal tract after contact with infected persons, through environmental contamination, or after ingestion of contaminated food or aerosols [86].

Although harmonization of laboratory methods was one of the keystone activities of the FBVE network, this has proven difficult to accomplish, as no consensus could be reached among laboratories with respect to the genomic target region. This is a general problem related to the diversity in demand for genetic typing methods [333]. For monitoring trends at the level of genotypes, resolution of sequence-based typing does not need to be very high, and this can be achieved by sequencing a relatively conserved genomic fragment [308]. However, the identification of epidemiologically linked patients requires sequence typing at a much higher resolution level. As this should be done by local public health laboratories with limited resources this, for the time being, excludes for instance the option to move to full-length genome sequencing. For norovirus in particular, a recent study made a start towards such a scientific basis by comparing two standardized ORF2 genotyping protocols in a small set of pre-selected norovirus strains [225]. However, with the rapid development of technology of both sequencing methods as well as computational tools [140], systematic analysis of large databases with both sequences and epidemiological data like the FBVE database is now possible. Here, we provide a method for identifying the most informative region of a pathogen, with NoV as an example, to guide future laboratory efforts in harmonizing pathogen typing methods.

Methods

Dataset

Selection of Sequences: We compiled 573 norovirus capsid gene sequences with background epidemiological data, as available from the public domain per April 1, 2010, representing the diversity of norovirus strains detected since 1999. Sequences were collected through the FBVE network (N=183), or drawn from Genbank (N=390). In all, these 573 sequences reflected 502 unique sequences.

Classification of Sequences: Genogroups, genotypes and variants were assigned according to capsid-based phylogenetic clustering, as described by Kroneman et al. (http://www.rivm.nl/mpf/norovirus/typingtool) [185]. A total of 77 sequences (52 unique) were defined as clusters of linked sequences, on the basis of available epidemiological and molecular information reported to FBVE, and published work [242, 281, 307, 342]. Two clusters (Table 1) represented known common source internationally dispersed outbreaks for which multiple strains of patients had been detected in different countries, i.e. 'event 1' with two II.1 sequences linked during the emergence of the polymerase-type II.b [107], and 'event 2' with eight II.4-2006b sequences linked during an international outbreak in Lourdes [281, 342]. Nine additional clusters consisting of different sequences detected in chronic shedders were found over a prolonged period of time within single patients. These were added to the dataset as linked sequences that may represent the diversity within outbreaks (Table 1, shedders 1-9). The nine shedder clusters consisted of 42 strains, i.e. II.3 (shedders 4, 6, 9), II.4-2004 (shedder 1, 3), II.4-2006a (shedders 2, 5, 8), and II.4-2006b (shedder 7) [242, 307]. We further refer to these 11 clusters as 'outbreak events'.

Phylogenetic Analysis

Full Alignment: Full capsid nt sequences transformed to amino acids (AA) and protein sequences were aligned using the default mode in MUSCLE version 3.6 [88]. Initial alignments for each genogroup were prepared separately, and then merged as profiles into the full alignment. The AA in the full alignment were converted back to the corresponding codon triplets. Tulane virus (EU391643) was included as out-group.

Sub-alignments: A script was written in Perl (www.perl.com) to prepare alignments of simulated PCR products of sequences of different lengths and targets alignments, i.e. sub-alignments, that were sliding windows of varying size (100, 200, 250, 300, 400, and 500 nt). To standardize window-taking from the gapped full alignment, a reference sequence was arbitrarily selected (a II.4 genotype strain; AB220921). Sub-alignments were taken to include the given number of nt from this reference sequence. The program was written to start in the middle position of the aligned reference sequence (for window 100: (1620–100)/2 = 760), then repositioned to both ends (for window 100: 1, and 1520), then repositioned to the two middles (for window 100: 760/2=380, and 380+760=1140) and so on, so that the windows would be evenly distributed over the capsid gene. This procedure yielded 7976 sub-alignments, of which 3075 were further analyzed.

Maximum likelihood (ML) Tree Building: a script was written to run ML tree building for the full alignment as well as for all sub-alignments in RAxML 7.0.4 [318]. This was done in the same order as the generation of sub-alignments, so that at each point in the analysis the tree-building would cover an even distribution of the capsid gene. ML tree building was done using the substitution model GTRGAMMA, partitioning the 3rd codon position from the other two codon positions (i.e. giving less weight to the 3rd codon position). Trees were built in several runs to balance the calculation time needed and the level of detail needed for representative results. The latter was checked in a higher number of sub-alignments for a single window.

Comparison of Trees

ML Tree Scoring: All ML trees were evaluated for their ability to cluster each group of a type (these types being genotypes, variants and outbreak events) in isolation from other groups of the same type. To do so, a script was written to calculate a branch impurity score for

each group type in each tree, using the bipartitions analysis in Phylip version 3.69, as follows. For each group, the smallest branch containing all sequences belonging to the group was identified; this can be called the 'complete branch'. The number of invading branches was counted, i.e. subbranches within the complete branch that consist only of sequences not belonging to the group. This invader count was normalized by dividing by the maximum possible invader count for the group, i.e. the number of leaves in the tree that are not part of the group. Such normalized invader counts were averaged for all groups of the same type, yielding a branch impurity score between 0 and 1 for the tree, with a score of 0 for the optimal case where no groups have any invading branches.

Tree Scores Comparison: The branch impurity score for each sub-alignment tree was plotted against its mid-position of the sub-alignment in the capsid gene, and for all window sizes in order to identify the optimal area of the capsid gene with the lowest impurity scores.

Validation

Bootstrap Analysis: In order to identify the optimal typing region, and to identify the optimal fragment length in the optimal typing region, non-parametric bootstrap values were calculated for the full-capsid gene tree as well as the sub-alignment trees for each of 6 window sizes for a selection of center positions within the identified area in the tree-score comparison step. In addition, bootstrap analysis were performed for ML trees based on the alignments which would be obtained when using genotyping protocols for genomic regions currently commonly applied [170, 225, 245, 349, 362] for region C, D, E, and the P2 domain. This analysis step thus resulted in bootstrap values for 43 ML trees. One hundred runs of exhaustive bootstrap analysis were performed using RAxML 7.0.4 (option -f i).

Branch Support Values: For each tree, all branch-supporting bootstrap values were identified supporting the complete branches of single genotypes (or variants, or outbreak events). A bootstrap value ≥70 was considered well performing [134]. Results were ranked into 6 categories, based on the branch-supporting bootstrap values and the number of invading sequences (Table 1).

Specificity Analysis: Specificity was considered 'able to cluster strains from a specific genotype (or variant, or outbreak event) together as a pure branch separated from others in the ML tree'. To evaluate the value of bootstrap analysis at different levels of resolution, the specificity was calculated as the percentage of genotypes (or variants or outbreak events) meeting criterium 1 (i.e. pure branches with bootstrap value ≥70) as well as for criteria 1 and 2 together (i.e. pure branches irrespective of the bootstrap values), with 80% being considered adequate specificity.

Results

Phylogenetic Analysis

Full Alignment ML Tree: The alignment covered a total of 1957 positions, with the longest sequence containing 1677 nt. The full alignment resulted in a ML tree capable of clustering all genotypes, 10/12 variants and all outbreak events grouped together but separately from other genotypes, variants and outbreak events (ML tree for separation of genotypes is shown in Figure 1).

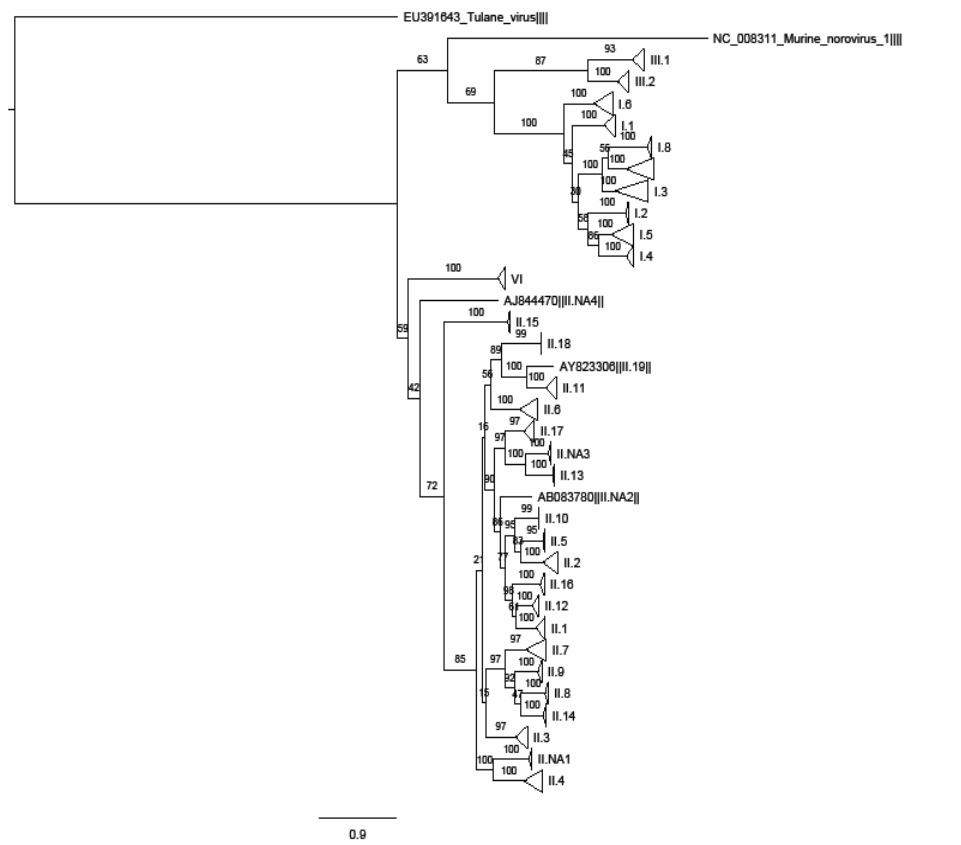

Figure 1. Maximum likelihood tree for 502 unique full capsid gene sequences from the Foodborne Viruses in Europe database (http://www.rivm.nl/pubmpf/norovirus/database#/outbreaks/list) and Genbank. Branches are condensed (triangles) to the genotype level, assigned according to the publicly available typing tool http://www.rivm.nl/mpf/norovirus/typingtool.

Sub-alignment ML Trees: In a preliminary run of 780 ML trees (130 for each window size), we found the window-100 performed poorly for segregation of variants and outbreak events, whilst window-250 clearly performed better (data not shown). In the second tree-building run, leaving out the window-100, and running extra analysis for window-250, a total of 2295 additional trees were built for evenly-spaced windows of 200 to 500 nt, including a higher frequency for window-250, for a total of 3075 ML trees for analysis: 130 window-100; 513 window-200; 959 window-250; 513 window-300; 513 window-400; and 513 window-500 trees. This yielded window spacing as low as every third nt for windows 200, 300, 400 and 500; the additional windows needed for every second nt added little information to the window-250, so the rest of the 7976 alignments were not analyzed.

Comparison of Tree Scores

ML trees were scored for the impurity of branches containing known clusters that indicates the ability to discriminate genotypes, variants, or outbreak events. The full-alignment tree performed very well, showing 100% segregation of strains belonging to distinct genotypes and outbreak events (score 0), and two invading branches for two variants (score 0.000759). Scores for sub-alignment trees were plotted per center position of the window along all nt positions of the full capsid gene (Figure 2). The top panel shows that for resolving genotypes, all regions across the gene perform well, even with the smallest (100-nt) windows.

However, for resolving variants or outbreak events, smaller windows perform distinctly worse, as do particular regions of the capsid gene. The gene region with center positions between 900 and 1150 was considered promising for resolving both variants and outbreak events (Figure 2).

Validation

Bootstrap Analysis and Branch Support Values: For 6 center positions within the promising region bootstrap analysis was performed. The region centered on nt position 1150 appeared optimal and was selected for further analysis. Table 1 shows 13 bootstrap results, with the six levels of the ability for a region to separate genotypes, variants and outbreaks given in gradations of colors. The full-alignment tree (column 1 in Table 1) clearly shows best

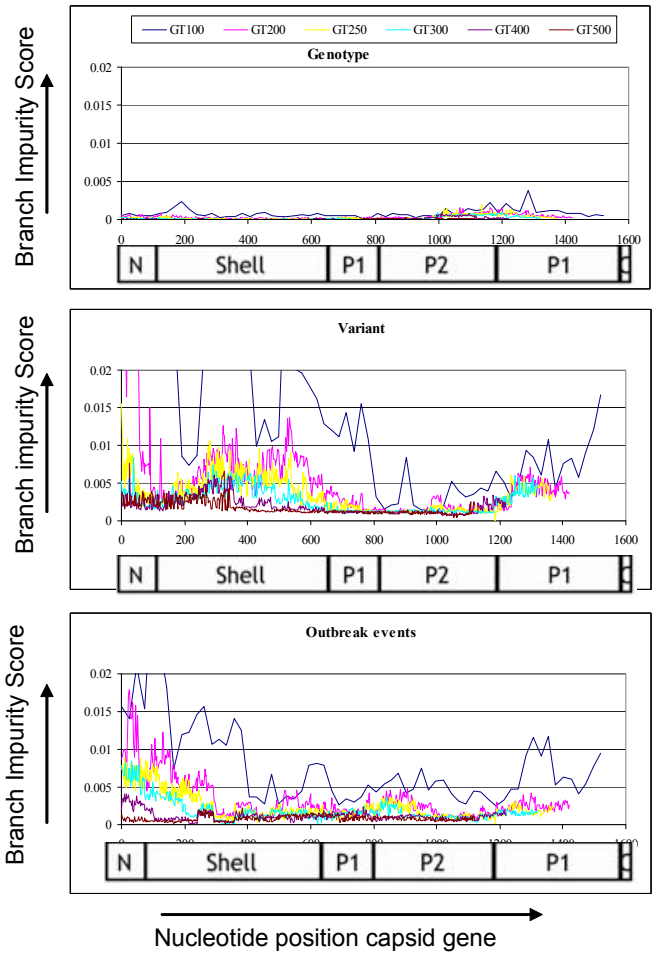

Figure 2. Summary of performance of phylogeny-based typing of norovirus capsid gene sequences. Branch impurity scores were calculated for each of 3075 maximum likelihood trees built in RAxML 7.0.4 [318] and presented at the mid position of the reference sequence AB220921. A score of 0 is optimal and indicates that all branches of a specific level do not show invading sequences within this sub-alignment tree; for example, all genotypes are correctly positioned together while separate from others. Scores >0 indicate that some of the complete branches within levels in the sub-alignment tree contain invading sequences. Scores were calculated for 6 fragment lengths, which are indicated as window 100 to window 500, and with each fragment length represented by a different color, and calculated separately for genotypes (upper panel), variants (mid panel) and outbreak events (lower panel). Scores for the full capsid alignment were 0 for genotypes, 0.000759 for variants, and 0 for outbreak events.

Table 1. Bootstrap values, as derived from 100 runs of maximum likelihood trees built in RAxML 7.0.4 [318], for branches without invading sequences or with a maximum of 1 invading sequence, and for different levels of resolution, i.e. genogroups, genotypes, variants and outbreak events. Bootstrap values were calculated for different fragment lengths within their optimal genomic region, and for several target regions in commonly applied genotyping protocols.

	N	1-1620 Full Capsid	900-1400 Window 500	950-1350 WIndow 400	1000-1300 Window 300	1025-1275 Window 250	1050-1250 Window 200	1100-1200 Window 100	1-282 G2SK region C	5-284 G1SK Region C	301-577 Region E	795-1253 P2 domain	1372-1585 CapC/D1/D3 R Region D	1439-1581 CapA/B1/B2 Region D
GGI	46	100	85	73	60	36		35	51		60	71	90	94
GGII	443	72								38				
GGIII	8	87	93	83	70	85	66	29	79	75	84	83	78	75
GGIV	3	100	99	98	98	89	94	93		85	100	100	97	97
Range		*72-100*	*85-93*	*73-83*	*60-70*	*36-85*		*29-35*	*51-79*	*38-75*	*60-84*	*71-83*	*78-90*	*75-94*
III.1	3	93	100	98	79	81	86	83	79	80	72	90	71	58
III.2	5	100	88	84			51	81			94	97		
I.6	6	100	92	100	83	86	80		61	63	97	95	74	
I.1	6	100	92	92	91	82	18	72	96	95	96	99	91	80
I.3	11	100	81	68	69	38					59	80		
I.8	2	100	100	100	99	92	94	90	97	95	100	100	98	94
I.7	3	100	75	75	70	73	71	84	77	72	61	93	36	93
I.2	6	100	91	86	63		92	48	99	99	96	98	100	96
I.5	3	100	100	72	65	39	30	63			79	72		
I.4	9	100	100	100	98	91	96	75	96	90	99	99	99	95
II.NA1	3	100	100	99	99	97	97	96	100	100	99	99	96	84
II.4	282	100	95	86	64	81	65	14	95	97	67	97	90	57
II.13	3	100	99	98	90	94	95		89	89	99	97	98	93
II.NA3	2	100	100	99	94	84	81	73	89	82	73	93	98	98
II.17	4	97	83	79	71	70	71	47	83	83	90	86	93	92
II.16	10	100	93		85	87	83	78	77	74	90	96	100	100
II.12	9	100	98	88	95	97	82	57	86	91	69	91	94	76
II.1	11	100	98	99	91	85	81	49	86	82	79	100	70	
II.10	3	99	99	99	97	98	97	87	97	98	99	96	100	98
II.2	9	100	87	97	97	98	88	68	97	100	83	89	99	89
II.5	5	95	89	96	95	92	91	86	99	99	67	81	82	72
II.6	12	100	100	98					91	95	98	97	79	55
II.18	2	99	88	100	98	96	95	100	100	100	100	100	5	4
II.11	4	100	98	95	89	81			94	91	86	94	70	81
II.3	58	97	68	63	41				80	75	89		90	92
II.9	3	100	82	75	23	27	24	10	96	95	97	84	94	86
II.8	4	100	96	94	85	86	93	81	90	95	89	98	100	97
II.14	8	100	92	88		84	79	78	77	79	97	88	94	75
II.7	7	97	90	72	25			60	89	91		100	69	61
II.15	2	100	98	97	83	85	60	84	99	100	98	92	97	96
Range		*93-100*	*68-100*	*63-100*	*23-99*	*27-98*	*18-97*	*10-100*	*61-100*	*63-100*	*59-100*	*72-100*	*5-100*	*4-100*

Table 1 – *continued*

	N	1-1620 Full Capsid*	900-1400 Window 500*	950-1350 WIndow 400*	1000-1300 Window 300*	1025-1275 Window 250*	1050-1250 Window 200*	1100-1200 Window 100*	1-282 G2SK region C*	5-284 G1SK Region C*	301-577 Region E*	795-1253 P2 domain*	1372-1585 CapC/D1/D3 R* Region D	1439-1581 CapA/B1/B2 * Region D
II.4-2006b	67			70	71	73	79		35		54		77	
II.4-2002/CN	4	86	38	41	40	4	24	3	57	70	66	71		
II.4-2003	19	100	99	94	92	91	80	86	73	77		100	54	5
II.4-2002	36	98	100	99	97	94	89	44			45	99		
II.4-2007	11	98	92	81	68		86	24	91			86		55
II.4-Camb	3											27		
II.4-2001	4	100	90	84	87		96	94	71	76	87	92	99	93
II.4-Bristol	4	100	100	91	89	90	83	99	100	100	78	100	100	100
II.4-1996	50	80							48					
II.4-2008	11	94	87	93	88	71		52	6			80	74	44
II.4-2004	31	85	80	68	10				40	10		67	86	8
II.4-2006a	39	85	73	65		49	31	4				61	51	
Range		*80-100*	*38-100*	*41-99*	*10-97*	*4-94*	*24-96*	*4-99*	*6-100*	*10-100*	*45-87*	*27-100*	*54-100*	*5-100*
Event 1	2	99	66	40	55				48	49	72	26	79	
Event 2	8	97	35	42	30	55	32				82	39	39	
Shedder 1	8	100	79	79	31	34	32				53	42		
Shedder 2	2	100	94	91	95	86	73	52				86	39	61
Shedder 3	2	100	96	89	91	87	84	95	48	32	99	92	63	
Shedder 4	3	100	95	100	99	93	95			56	92	99	42	70
Shedder 5	7	99	93	85	84	59	66	61			78	94		
Shedder 6	4	92	63	54	63	97	91	57	79	78	92	77		51
Shedder 7	2	94	6	69	55	61	58	60			52	71	51	57
Shedder 8	2	98	96	85	94	97	94	86	50	52	37	95	70	63
Shedder 9	12	99	88	93	74			76	54		84	93	93	89
Range		*92-100*	*6-96*	*40-100*	*30-99*	*34-97*	*32-95*	*52-95*	*48-79*	*32-78*	*37-99*	*26-99*	*39-79*	*51-70*

Different colors indicate different levels for mismatching of branches into a genotype, variant or outbreak event, as follows:

1 Pure branch with bootstrap value>70

2 Pure branch with bootstrap value <70

3 Impure branch with one invasion sequence and with bootstrap >70.

4 Impure branch with one invasion sequence and with bootstrap <70

5 Branch is split in groups, or impure including 2 or more invasion sequences from other genotypes (or variants, or outbreak events), i.e. others of same level; branch support value unresolved

6 Branch is split in groups, or impure including 2 or more invasion sequences from other genogroups (or genotypes, or variants), i.e. others of a higher level; branch support value unresolved

results for all levels of pure branches with bootstrap values ≥70 (criterium 1) and pure branches irrespective of the bootstrap values (criteria 1&2). With respect to the sub-alignments the window of 500 NT, i.e. positions 900-1400, is best approaching the full-alignment tree on the basis of criterium 1 as well as criteria 1&2 together. This region of the capsid gene is best able to correctly cluster genotypes, variants and outbreak events simultaneously, while showing invaders in 3 variants and one outbreak event. With respect to the commonly used PCR regions, the P2 domain shows best results for pure branches with bootstrap values ≥70 (criterium 1), and also for pure branches irrespective of bootstrap values (criteria 1&2).

Specificity: Table 2 shows specificity for each of 13 genomic regions in recognizing genogroups, genotypes, variants and outbreak events as groups separated from other sequences in their ML tree. The full capsid ML tree clearly shows optimal performance, with 100% specificity for typing of genogroups, genotypes and outbreak events, and 83% specificity for typing of variants. The window-sizes with the center being the 1150th nt approach this optimal performance, with window 500 showing best performance for genogroups, genotypes, variants, and outbreak events simultaneously. Window 400 can be considered the minimum fragment length still able to recognize outbreak events included in this analysis as pure branches with adequate specificity (i.e. >80%) if low bootstrap values are considered acceptable. The specificity for variants in windows 500 on the basis of criteria 1&2 can be increased to 83% if recognition of variants is based on a shorter fragment within this region, thereby obtaining the same specificity as the full capsid ML tree.

Table 2 also shows that the P2 domain is the best performing PCR currently available for simultaneous recognition of genotypes, variants and outbreak events. On the contrary, in >50% of the outbreak events, unrelated strains will be considered part of the outbreak event when using the commonly applied primers for region C or D as a standardized method. The PCR for region E showed adequate performance in recognizing outbreak events, but low in recognizing variants.

Overall, results of the full alignment are maximal for recognizing outbreak events. Its performance can be closely approached on the basis of pure branches, irrespective of bootstrap values, in ML trees in the 900-1400 region, while the 950-1350 region can be used for the II.4-2006b variant. Lack of specificity is mainly caused by low bootstrap values for pure branches (i.e. <70, criterium 2). However, despite these low bootstrap values, the known clusters appear as pure branches in the ML trees.

Discussion

Within the available norovirus capsid genes, we identified nt positions 900 to 1400 as the informative genomic regions best approaching the full capsid sequence in its ability to correctly assign genotypes, variants and the outbreaks events used in this analysis simultaneously. The positions 950 to 1350 of these norovirus capsid genes can be considered the target and minimum fragment length for laboratory networks aiming to identify outbreak events with specificity>80%. With respect to the remaining currently available protocols [170, 225, 245, 349] we found that the P2 domain best approaches the full capsid sequence. Region E (nt 301-577) tends to mainly have difficulty to distinct variants as pure branches from other branches, whereas PCRs for regions C (~nt 1-284) and D (~nt 1372-1585) tend to

have difficulty to distinct at least one third of the selected outbreak events as pure branches. Both the P2 domain and the newly identified optimal fragment are in the variable genomic region, and genogroup- or genotype-specific primers might be needed. Since the fragment is located between regions E and D, primers are in theory available for the ~600 to 1400 nt positions.

Table 2. Specificity (%) of different genomic regions in clustering genogroups, genotypes, variants and outbreak events as a group separated from others, as derived from bootstrapped Maximum Likelihood trees*

		Full	Windows (center 1150)						PCR regions					
Group	N	1-1620 (1620 nt) Full Capsid	900-1400 (500 nt) Window 500	950-1350 (400 nt) Window 400	1000-1300 (300 nt) Window 300	1025-1275 (250 nt) Window 250	1050-1350 (200 nt) Window 200	1100-1200 (100 nt) Window 100	1-282 (282 nt) G2SK region C	5-284 (280 nt) G1SK Region C	301-577 (277) Region E	795-1253 ((459 nt) P2 domain	1372-1585 (214 nt) CapC/D1/D3 Region D	1439-1581 (143 nt) CapA/B1/B2 Region D
On the basis of criterium 1: Pure branches with support values >70														
Genogroups	4	100	75	75	50	50	25	25	25	50	50	75	75	75
GI genotypes	8	100	100	88	63	63	63	38	75	75	75	100	63	63
GII genotypes	20	100	95	90	70	80	65	45	100	100	80	95	63	75
GIII genotypes	2	100	100	100	50	50	50	100	50	50	100	100	50	0
Variants	12	83	67	58	50	42	50	25	33	33	17	58	42	17
Outbreak events	11	100	64	64	55	45	45	27	0	0	55	64	27	9
On the basis of criterium 1 and 2: Pure branches irrespective of support values														
Genogroups	4	100	75	75	75	75	50	75	50	75	75	75	75	75
GI genotypes	8	100	100	100	100	88	88	75	75	75	100	100	75	63
GII genotypes	20	100	100	95	85	80	75	70	100	100	95	95	100	95
GIII genotypes	2	100	100	100	50	50	100	100	50	50	100	100	50	50
Variants	12	83	75	83	75	58	67	67	67	33	42	75	58	50
Outbreak events	11	100	91	82	73	64	64	64	36	36	82	73	45	36

*Nucleotide position was chosen from the reference strain AB220921, and nucleotide position 1 of the capsid gene corresponds with nucleotide position 5085 of the GII-4 strain Lordsdale/1995/UK (Genbank X86557)

Non-parametric bootstrapping, i.e. randomized selection of columns in the alignment, is commonly accepted as a method for assessing confidence of phylogenetic analysis [134]. It was proposed in 1985 by Felsenstein [98] as a method for obtaining confidence limits on phylogenies. Some columns in the alignment may be excluded, whereas others may be included multiple times, while adding up to the same number of nt positions included in the analysis. Although users of bootstrap values would like to use it to measure the 'truth', Felsenstein (1985) explicitly stated that *bootstrapping provides a confidence interval that contains the phylogeny that would be estimated from repeated sampling of many characters from the underlying set of all characters, NOT the true phylogeny* [98, 315]. Our analysis shows that pure branches in a ML tree may reflect the 'true' phylogeny irrespective of their bootstrap value. This is especially true for the variants and outbreak events. These pure branches were found in ML trees on the basis of sub-alignments, and could be confirmed in the full capsid sequence alignment ML tree, where bootstrap values were well over 70. The low bootstrap values can be understood when considering the low number of informative sites at these levels of resolution, where few mutations may be informative of the common ancestor. Randomization may thus exclude the informative sites, which will have a stronger effect on closely related strains in outbreak events or variants, when compared to genotypes or genogroups.

We caution against over-interpretation of these results. The approach chosen provides a method for comparing quality of results and weight of the conclusions drawn on the basis of the use of different genotyping targets. However, although the dataset represents a rather comprehensive collection of sequences, it is still rather small. Noroviruses that are selected for full capsid sequencing may not represent the general norovirus population. Most of our known clustered sequences (to represent outbreaks) were from chronic shedders, in which viruses may accumulate mutations [307]. This may explain the fact that the region subject to selective pressure was again identified as the most informative region for grouping outbreak strains together [362], albeit that Xerry et al. considered strains with one or more mutations in this hyper variable region as representing unrelated transmission events. Still, random mutations will remain informative in linking outbreak strains. It will be interesting to see if the P2 domain and nt 900-1400 remain the informative regions during prospective analysis of outbreaks with systematic full capsid sequencing of all patient samples, and compared to a set of strains randomly selected for full capsid sequencing.

The current analysis was only possible through the availability of the systematically collected FBVE data. Nevertheless, our findings may have consequences for such networks' conclusions with respect to the identification of international outbreak events. For example, in the FBVE network, 98% GI and 7% GII partial capsid sequences covered region E, whereas 85% of GII sequences covered the conserved region C and 2% of GI sequences region D [344]. In Calicinet, a US network aiming to detect outbreak events, the conjunction between ORF1 and ORF2 (i.e. region C of the capsid gene) and region D are considered best practice for norovirus detection as this region is highly conserved [156, 225]. Thus, if no secondary typing protocol was used, these networks are likely to have not recognized outbreak events within the background sequence noise. In Noronet, a network aiming to detect emergence of new variants, regions C and D of the capsid gene were used for collection of representative sequences. However, for example, variant assignment needed to be based on the full capsid sequence [308]. Future confirmation of our findings is likely to serve laboratory efforts in

identifying outbreak events by cutting down the number of clustering sequences to the most likely related ones.

In our aim to develop a generic method that should be applicable to multiple sequence-based datasets of other pathogens as well, we included a large variety of norovirus sequences in the alignment, i.e. all 5 genogroups. Alignments within genotypes, however, will logically show better performance of the ML trees. Therefore, aside from this method, in real-time analysis for confirmation of an outbreak event, additional phylogenetic analysis should be performed. Nevertheless, the consensus typing region for laboratories should be able to distinguish outbreak events irrespective of the genogroup, genotype or – new – variant involved, as this information is not known beforehand.

Outbreaks caused by sewage contaminated foods may involve multiple strains of different genotypes [196], which may be missed if linking is only focused on the identification of closely related strains. However, each of these multiple strains is likely to evolve within outbreaks as well, and phylogenetics may still add to the identification of such outbreaks. Nevertheless, the epidemiological focus should not be omitted in order not to miss outbreak events involving multiple genotypes.

The here proposed method may be applicable to other pathogens for which full length sequences together with epidemiological information are available. Despite the calculation time needed for this method, and the development towards whole genome sequencing, we are of the opinion that our method will remain of use for public health purposes. The analysis can be performed as an evaluation point to guide laboratory efforts in recognizing international outbreaks once a large enough dataset of reference sequences of substantial length is available. Although the costs of whole genome sequencing are decreasing, obtaining a full genome sequence of pathogens that cannot be cultured will remain a challenge for regional diagnostic laboratories. Therefore, using shorter fragments sufficiently specific in recognizing outbreak events as a consensus is likely to improve the identification of international outbreak events.

Viruses Transmitted through the Food Chain: a Review of the Latest Developments

Chapter 9

Linda Verhoef, Ingeborg L. Boxman, and Marion Koopmans

CAB Reviews 2008;3:e-pub.

Abstract

The emergence of severe acute respiratory syndrome (SARS), Nipah virus and H5N1 has sparked fears of foodborne dissemination of viruses, resulting in an increased focus on microbiological food safety. Norovirus (NoV) and hepatitis A virus (HAV) have been recognized as priorities based on their illness burden, and as models for prevention studies. In addition, hepatitis E virus is considered an emerging foodborne pathogen. Systematic surveillance for foodborne viral diseases, where present, has to deal with underreporting, with available data biased towards developed countries, making true estimates of the burden of illness challenging. Nevertheless, foodborne viral outbreaks are often reported, whereas current measures undertaken by food safety management organizations are focused primarily on bacterial contamination and are insufficient for viruses in food. There has been a shift towards molecular techniques for the diagnosis and detection of viruses in food, but a definite link to food is still hard to establish owing to the low levels of viruses in foods. Moreover, multiple strains may be involved in sewage contaminated foods, with the risk of recombination and development of novel strains after ingestion. To enable monitoring of food and food safety, sensitive routine methods are needed, and methods for detection of NoV and HAV in risk foods (shellfish, soft fruit and leafy greens) are currently validated. Norovirus cannot be cultured, but the murine NoV may be a surrogate for studying NoV survival. In all, significant progress has been made in understanding foodborne viral infections, but clear data gaps still exist and should be the focus of future research.

Introduction

Illness caused by the consumption of food contaminated with viruses has long been recognized as a phenomenon, but has continued to be somewhat of an obscure topic in the food safety debate. In recent decades, the world has witnessed increasing focus on microbiological safety of food for consumers, with targets set by the International Codex Alimentarius [14]. Nevertheless, the remarkable reality is that even in 2008, after the scares of bovine spongiform encephalothapy (BSE) [274], avian influenza [6] and severe acute respiratory syndrome (SARS) [7] as foodborne pathogens, there are no control measures that have been specifically designed for control of viral foodborne illness. The widely held standard for microbial safety of food, i.e. the presence of less than the European legislation limit of 230 most probable number (MPN) of *Escherichia coli* per 100 g (ISO 16649-3), has very little correlation with the presence or absence of viruses. The literature of recent years contains a long list of reports of well-documented viral foodborne outbreaks, reflecting an increasing awareness of this topic in public health settings and in the research community. Here, we review current information on the viruses involved, estimates for the burden of foodborne illness, progress and challenges to detection and data gaps needed to be filled before action can be taken by food safety management organizations.

Viruses Involved in Foodborne Transmission

Noroviruses (NoVs) and Hepatitis A Virus (HAV)

Currently, the most common foodborne virus problems are caused by human viruses that infect and cause illness after ingestion and are shed via stool. Transmission may occur through sewage contamination early in the food chain or lack of personal hygiene later in the food chain, particularly when ready-to-eat (RTE) food-items are involved. An expert meeting organized by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) concluded that NoV and HAV in shellfish, fresh produce, and prepared foods were priorities [18]. These viruses are considered to be associated with the greatest burden of illness, and are also seen as models for studying factors determining success of prevention of foodborne spread. NoVs are RNA viruses that cause gastroenteritis (GE) within 12–72 h after exposure. They overshadow other foodborne viruses in the literature because of their etiological dominance, infectiousness and diversity. Currently, 5 genogroups (G) have been described, subdivided into at least 40 genetic clusters [120, 174]. In addition, new variants within the common GII.4 genotype have emerged every other year worldwide since 2002 [1, 182, 205, 341]. NoVs are transmitted by person-to-person contact, environmental contamination, and food- and waterborne transmission. The foodborne proportion of the infections was estimated to be 40% in the USA [229] and 25% in Australia [126]. In Europe, 22% of outbreaks are thought to be foodborne [343], but underreporting is likely [247]. Recently, a common human NoV variant, GGII.4, was detected in pig faeces and in meat, suggesting the potential for zoonotic transmission [224]. NoV generally causes mild disease, but recent epidemics have been associated with higher mortality among the elderly [20].

HAVs cause hepatitis 2–6 weeks after exposure. This is far less common than NoV gastroenteritis but more severe, particularly with increasing age [36]. Unlike many RNA viruses, the HAV genome is highly conserved with 3 genotypes of strains infecting humans [64], and no known zoonotic transmission. Owing to the long and variable incubation period, underreporting of outbreaks and sources is likely as patients need to recall their food exposures 2–6 weeks before onset of disease. For this reason, the proportion of infections to be attributed to food is difficult to determine, but nevertheless estimated to be 5% in the USA [229]. The risk of outbreaks of HAV is changing because endemic circulation has become less common and therefore the population as a whole is becoming more susceptible [174]. Rotaviruses, astroviruses, enteric adenoviruses, Sapporo-like viruses (SLVs) can be transmitted via food [74, 106] but are less common.

Emerging (Foodborne) Viruses

Since 2001 several new potential foodborne virus problems have been identified, in the process highlighting the (increasing) complexity of our globalizing food chain, and the challenges that this poses to health protection. The emergence of SARS, Nipah virus and H5N1 avian influenza has sparked fears of foodborne dissemination of these serious health threats, with anecdotal evidence that such fears could be based on fact [6, 7, 213]. Hepatitis E virus (HEV) has been added to the priority list of foodborne viral infections. Here, historically, genotypes 1 and 2 were known as causes of hepatitis in travelers to developing countries, but recently a third genotype of HEV (gt 3 HEV) was identified that is widespread in pigs across the world, with some human cases found in regions previously thought to be free from HEV [268]. Waterborne transmission is the most commonly reported mode of

transmission in developing countries [268], but a cluster of gt 3 HEV cases in Japan was linked epidemiologically and genetically to the consumption of undercooked pig livers and deer meat [325, 367]. The consumption of raw pork sausages has been postulated as a possible source of infection in the Netherlands but without convincing evidence [230]. The gt 3 HEV virus can be found in meat on the market, suggesting potential for zoonotic transmission [30, 96, 197]. Underreporting of HEV genotype 3 infections is likely, as it is a newly recognized pathogen outside the tropical regions with a long incubation period. The proportion of infections attributed to food is currently unknown, but secondary transmission is relatively uncommon. HEV usually causes mild disease, but mortality up to 20% has been reported among pregnant women [120]. Finally, Aichi virus is an emerging virus with foodborne transmission potential. Since its discovery in 1989, it is mostly associated with oyster-related non-bacterial GE, and associated with disease in Japan [366] and France [12]. Two genetic clusters were recognized, based on 17 strains [366]. In a study systematically testing for Aichi virus, it was not detected as a causative agent of GE [323]. As soon as more literature becomes available, Aichi may need to be added to the priority list of foodborne viral infections

Methods for Detection of Foodborne Viruses

Foodborne Virus Detection in Humans

Methods for the diagnosis of viral infection differ widely between countries [204, 348]. Asymptomatic shedding has been repeatedly reported for NoV [104, 115] and HAV [100, 317], complicating diagnosis. NoVs are usually detected in stool, particularly in outbreaks [71, 87]. Over the last decade, methods for detection have shifted from the gold standard of EM to molecular techniques with consequent broader applicability, and potential for higher sensitivity [204] and international comparison of strains [348]. Routine laboratories may not currently be able to perform these methods, but that situation is changing rapidly. For HAV and HEV infections, human serum is tested for the presence of antibodies [120], with the consequent diagnosis of individual illness. In Europe, most countries have at least one specialized laboratory capable of detecting HAV antibody in human serum and NoVs in stool [204]. Real–time reverse transcription polymerase chain reaction (RT-PCR) amplification techniques allow for quantitative analysis [217] and have been described for NoV [81, 138, 139, 156, 157, 232, 253, 273, 296, 330], HAV [58] [59, 141, 152], HEV [91, 154, 220, 251, 258]; adenovirus [132, 151, 202], astrovirus [122, 284], and rotavirus [10, 202, 231, 254, 299].

Detection of Viruses in Food

The detection of viruses in food has its own challenges, because often only low numbers of viruses are present in the food, which have to be extracted and concentrated. In addition, viruses may be heterogeneously distributed in food in which viruses do not grow. Localization of viruses in the food will be at the surface (RTE foods, produce), in digestive glands of shellfish [191] or in tissues in the case of zoonotic transmission [325, 367]. Recently, evidence was found for uptake of HAV through contaminated irrigation water inside green onions [48]. Matrices for which methods have been developed with some success are shellfish and fresh produce [21, 117].

Cell-culture-based assays can discriminate between infectious and non-infectious viruses [72], but to date are not available for human NoV [85]. Instead, feline calicivirus (FCV)

has often been used as surrogate in inactivation studies [223]. FCV is, however, a respiratory virus, which is, for example, more sensitive to pH changes as compared with NoVs transmitted via the faecal–oral route. Recently, the first in vitro NoV cell culture model was reported for a NoV that infects mice [360, 361], which now is being widely explored as a surrogate model for studying environmental survival of human NoVs [45, 237]. Also, an in vitro cell culture infectivity assay for human NoVs was reported, although this is not yet widely applicable [320].

Real–time RT-PCR amplification techniques for detection in food or environmental samples have been described for NoV [33, 153, 203, 240, 243], HAV [5, 35, 59], HEV [154] and astrovirus [189] [122]. Assays for detection of viruses in foods are mainly done in specialized laboratories, with routine testing restricted to monitoring of shellfish. Many methods have been developed for the concentration of viruses from bivalve molluscs or from the shellfish digestive organs only (reviewed in [117], [192, 198, 302]). Methods are not standardized, although some comparative studies have been done [57, 297, 322]. Viral detection from soft fruit (raspberries and blackberries) can be hampered by the presence of inhibitors in extracts, release of acid juices and the formation of a pectinaceous gel-like substance [43]. Therefore, elaborate extraction methods are needed, in which viruses are separated from the products, concentrated and subjected to a process that removes inhibitory substances [43, 82, 84, 123, 195, 286, 287, 300]. The CEN/TC275/WG6/TAG4 committee of the European Union is validating standardized sensitive methods for NoV and HAV detection in selected food matrices (bivalve molluscs, soft fruit, leafy greens and bottled water), including process controls [59].

Finally, there is another, quite challenging, category, consisting of any other foods that can be contaminated during handling by an infected food handler (RTE foods). Studies investigating RTE foods mainly have focused on deli-meat products, sometimes as a part of outbreak studies [33, 148, 218, 298]. Recently, we explored the use of environmental swabs for the investigation of NoV foodhandler-related outbreaks [31]. Analyses of swabs taken from surfaces of kitchens and personnel bathrooms revealed the presence of NoV with identical sequences to those detected in clinical specimens from ill people in outbreaks linked to restaurants. Environmental swabs may become an important tool in outbreak investigations to inform of the potential transmission route.

Monitoring

Foodborne Illness

The increasing awareness of GE [63, 108] and foodborne (viral) illness [4, 17, 95, 228, 236] is reflected in the literature but with a bias towards developed countries [17]. Despite earlier recommendations [172], systematic surveillance for foodborne viral disease other than NoV outbreaks [174] has not yet been achieved, or – where present – has to deal with considerable underreporting [236]. In part, this is explained by the difficulty in linking illness to food consumption when the incubation period is long (HAV and HEV) [120], when more than one possible route of transmission is possible, and when detection is difficult due to the variability of viruses (NoV) [71, 348]. Viruses are likely to be an important cause of unexplained GE [229], and food may be an important vehicle [100]. The latter was acknowledged by, among others, the Foodborne Viruses in Europe (FBVE) network

Table 1. Examples of epidemiological studies investigating illness associated with foodborne viruses

Method	Country	Study period	Focus	N	Illness	Viral etiology	Analysis clinical* samples	Food* analysis	Remarks	Reference
Surveillance										
Reported outbreaks	Austria	2005	Foodborne outbreaks	606	Range	<1%	Yes	No	Data suggestive for insufficient quality of outbreak investigation	[236]
Reported GE outbreaks	US	2001-2004	Cruise ships	14,842	AGE†	Not specified	No	No		[63]
Reported non-bacterial GE outbreaks	US	1997-2000	Non-bacterial outbreaks	284	NoV	93%	Yes	No	57% reported foodborne	[95]
Risk estimates using data from surveillance and longitudinal study	England and Wales	1996-2000	Cases of indigenous foodborne disease	>1.7 million (estimated)	Range	5%	Yes	No		[9]
Viral GE	Europe	1995-2000	NoV	3,714	NoV	100% and >85% NoV	Yes	Yes	% foodborne differs between countries	[209]
Survey of health departments	Australia	1995-2000	Summary of epidemiology of foodborne disease	293 outbreaks	GE†	4%	Yes	No		[68]
Reported GE outbreaks	US	1980-1994	Seafood related outbreaks	339	range	42%	Yes	No		[352]
Community-based analytical studies										
Intensified outbreak surveillance	Netherlands	2002	GE outbreaks	281	GE	56%	Yes	Yes	21% of all GE outbreaks foodborne	[336]

Community-based prospective cohort	Netherlands	1999	Incidence GE	283/1000 PY[§]	GE	21%	Yes	No	First recognition of importance of viruses	[73]
Population-based community cohort	England and Wales	1993-1996	Incidence and etiology of IID[‡] cases	194/1000 PY	IID	29/1000 PY	Yes	No	Considerable underreporting, varying between pathogens	[355]
Outbreak studies										
Outbreak investigation	United States	2003	HAV outbreak due to green onions	601	hepatitis	100%	Yes	No	Largest documented HAV outbreak in the US. Apparent contamination of green onions before or during packing.	[354]
Review	Worldwide	1970-2003	Foodborne outbreaks on passenger ships	50	Range	8%	Yes	Yes		[282]
Reported NoV outbreaks	Japan	1997-2002	Molecular epidemiology	66	NoV	100%	Yes	No	No. of NoV genotypes depends on transmission route	[157]
Review	US	1968-2003	Foodborne HAV	31 outbreaks	HAV	100%	Yes	Yes	3/31 reviewed outbreaks with detection of viruses in food	[100]
Diffuse outbreaks										
Retrospective cohort	Australia	Nov 2003-Jan 2004	Source tracing leads to oysters	3 outbreaks 83 cases	NoV	100%	Yes	Yes	Geographically dispersed and multiple strains	[353]
Retrospective cohort	US	May 2002	Source tracing leads to wedding cake	46 outbreaks 2700 cases	NoV	100%	Yes	No	Ill food-handler and manually handled food	[104]
Retrospective cohort	Sweden	March 1999	Source tracing leads to pumpkin salad	30	NoV	100%	Yes	No	Single caterer for 30 day-care centers	[116]

*clinical samples positive and food samples positive; [†]AGE=acute gastroenteritis GE=gastroenteritis; [‡]IID=infectious intestinal disease; [§]PY=person years

Table 2. Summary of reports in which bivalve molluscan shellfish were monitored for viral contamination

Source of Product	Period	Samples analyzed	positive NoV*	positive HAV	positive EV	positive other	Reference
Thailand	2005-2006	120				3% RV	[166]
Italy	2005	235	14%	6%			[67]
India	2003-2005	174	[†]		41%	21% AdV	[331]
Japan	2002-2005	1512	5%				[244]
China	2004	128	19%				[179]
Netherlands, Ireland, UK	2004	42	17%		5%		[32]
Netherlands	2003	64			22%		[201]
South-Korea	2002-2003	55			11%	89% AdV	[54]
USA	2002-2003	45	20%				[50, 60]
Norway	2000-2003	681	7%			19% AdV	[240]
Japan	2001-2002	191	9%				[243]
11 countries	2000-2002	507	11%				[50]
Tunisia	2000-2001	23	25%	26%	4%	61% AV	[89]
France	2000-2001	52			40%[‡]		[83]
France, Netherlands, Ireland	2000-2001	87	9%		13%		[26]
Greece, Spain, Sweden, UK	2000-2001	475	6% GI, 8%GII	2%	18%	39% AdV	[103]
Greece, Spain, Italy	1999-2000	290		18 % (34%[4])			[52]
Brazil	1999	27		22%			[56]
South America	1999	17		24%[3]			[279]
Spain	1998-1999	60		24%	19%	47% AdV	[238]
Italy and import	1998	170		14%			[216]
France	1995-1998	181	28%	5%	30%	30% AV, 37% RV	[190]
Spain	[¶]	164		27%	44%		[280]
Italy	[¶]	142		35% (13%[§])			[72]
Italy	[¶]	36		36 %	14%		[66]

*NoV=norovirus, HAV=hepatitis A virus, EV=enterovirus, AdV=adenovirus, AV=astrovirus, RV=rotavirus; [†]blanks were used when samples were not evaluated for the designated virus; [‡]from production areas associated with outbreaks; [§]proportion that was positive in cell culture; [¶]period not specified

Table 3. Examples of outbreak studies successful in linking viruses in suspected food with clinical specimens

Virus	Food matrix	Year of outbreak	Country	Source of product	Linking by sequences	References
NoV	Fruit cake, chocolate éclair	2006	Netherlands	Food handler	Yes, identical	Boxman unpublished data
HAV	Oysters	2005	USA	USA	Yes, identical	[303]
NoV	Oysters	2004	Netherlands	Not described	Yes identical	[276]
NoV	Deli-meat	2004	Netherlands	Food handler	Yes, identical	[33]
HAV	Sandwiches	2002	Italy	Food handler	Yes	[53]
HAV	Blueberries	2002	New Zealand	New Zealand	Yes, identical	[44]
NoV	Mussels	2002	Italy	Italy	Yes, identical	[266]
NoV	Oysters	2002	Italy, France	France	Yes, identical, multiple strains	[196]
HEV	Pig liver	2001-02	Japan	Japan	Yes, identical	[367]
NoV	Raspberries	2001	Sweden	import	(Yes) uncertain	[194]
NoV	Oysters	2001	Netherlands	France	Yes, identical	[32]
NoV	Ready-to-eat food (lunch)	2001	Japan	Food handler?	Yes, 96-99% similarity	[167]
NoV	Oysters	2000	France	France	Yes, identical	[193]
HAV	Shellfish	1999	Spain	Peru	Yes, identical	[29, 289]
NoV	Oysters	1999	New Zealand	New Zealand	92% match	[309]
NoV	Oysters	1998	USA	USA	Yes, identical	[302]
NoV	Deli ham	1998	USA	food handler	Yes, identical	[298]
NoV	Raspberries	1997	Canada	Bosnia	Yes, identical	[112]

[204, 348], resulting in a harmonized molecular monitoring system that contributed to the recognition of newly emerging NoV strains causing outbreaks worldwide [1, 182, 205, 341]. Despite the difficulties, outbreak surveillance has led to several interesting findings mainly involving NoVs (Table 1).

Food

For shellfish, monitoring is now performed in most parts of the world (Table 2), and this has revealed the inadequacy of depuration to eliminate viral contamination, as it does for *E. coli* [103]. Hence, viruses are frequently detected in shellfish from both commercial and non-commercial harvesting areas, and in products on the market for consumption (Table 2). For foods other than shellfish, the prevalence of viral contamination is unclear and data are limited to confirmation of viral presence in foods related to outbreaks [285, 352].

Data from Epidemiological Studies

Prospective Epidemiological Studies

Ideally, risk assessments have input from community-based prospective analytical studies. However, such studies are costly and therefore rare (Table 1) [17]. Nevertheless, a few studies have provided crucial population-based estimates for (viral) foodborne GE. Viral pathogens were found to be the most important causes of GE, with poor food-handling hygiene associated with NoV and rotavirus infections [74, 336].

Outbreaks

Recent outbreak reports [75, 115, 129, 135, 239, 292], reviews [121, 282, 329], and surveillance systems [95, 183, 236] mostly involve NoV and HAV. Often, multiple strains are found [110, 292], indicating sewage contamination [25, 352]. Descriptions of some geographically dispersed outbreaks [70, 116, 264, 310, 353] illustrate the difficulties of linking cases and food in space and time especially when frozen RTE foods are involved. Based on analytical and descriptive outbreak investigations, several products should be added to the list of foods requiring careful selection and handling to prevent foodborne disease: these are (frozen) berries [97], fresh-cut green leafy vegetables [228], and multi-ingredient foods [104, 121] for NoVs, shellfish and green onions [100, 265, 329, 354] for HAV, and undercooked deer [326] and pork meat [230] for HEV.

Linking Food and Human Data

The identification of identical strains in food and ill people can provide definite evidence for a causal relationship if properly validated. The problem is that virus strain identification in foods has rarely been achieved owing to food matrix complexity, low levels of contamination, genetic variability [192], and low availability of leftovers for analysis. Still, there are some successes (Table 3). Here, an important data gap is knowledge of the prevalence of strains in the environment, foods and humans as background for the interpretation of matching. This gap complicates the identification of a causative relationship between food consumption and illness.

Burden of Foodborne Viral Illness

Several research groups have made efforts to estimate the burden of foodborne illness at national [8, 73], regional [126, 229] and global [17] level, with estimates for viral illnesses

mainly available for developed countries and varying between 1 in 780 UK inhabitants and 1 in 33 US inhabitants [8, 73, 126, 229]. An expert meeting on viruses in food indicated the data gaps for viral foodborne infections worldwide, with analytical data mainly lacking in developing countries [18]. Underreporting of foodborne viral illnesses is the main shortcoming of surveillance systems and burden estimate calculation, which is illustrated with the surveillance pyramid (Figure 1) [17, 246, 355].

Challenges

The main challenge for foodborne viral illness lies in the furnishing of proof and causality. Given an unknown background level of viruses in foods, the environment or (asymptomatic) shedders, linkage of strains needs to be interpreted with caution and combined with epidemiological data. To enable monitoring of food, detection limits of assays should be established and should be low enough to detect viruses in naturally contaminated samples. Methods for measuring or approximating viability of a virus detected in food are needed to measure the survival [223] or inactivation by, for example, high hydrostatic pressure [301], heat treatment [65, 313], or UV [99].

Another important challenge is the rapid changing of virus strains, and their potential to generate recombinants in humans after simultaneous infection [14, 292]. Recent molecular data have shown that mixtures of viruses appear fairly common in shellfish [198] and sewage contaminated foods [110].

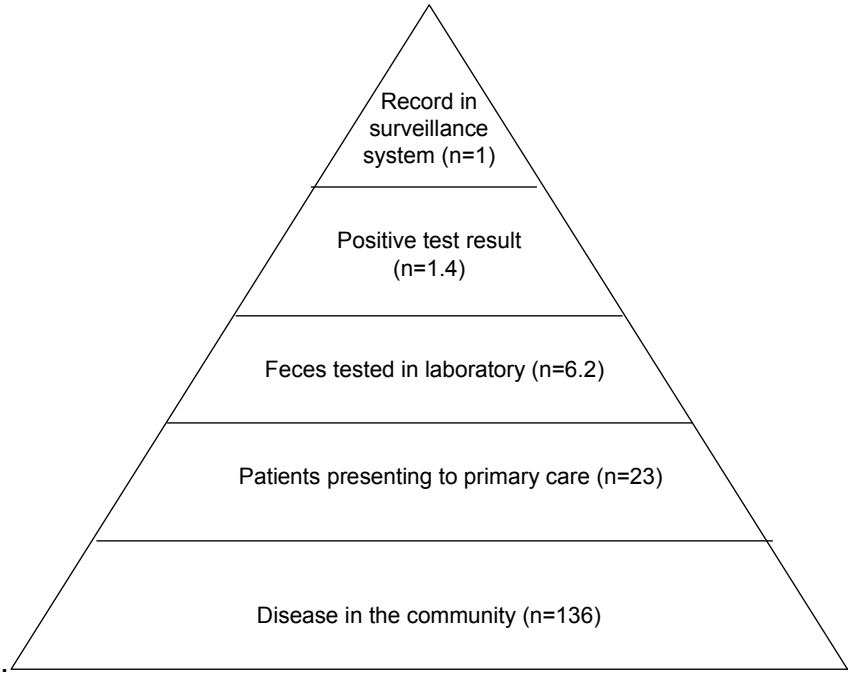

Figure 1 (adapted from O'Brien, 2008 [246] using data from Wheeler et al., 1999 [355]). For each case recorded in the national surveillance system, an estimated 136 cases are present in the community.

Prevention and Intervention

Many recommendations have been reported for shellfish and manually handled foods in the last decade based on epidemiological [15, 97, 104, 239, 249, 295, 310] and laboratory-based [39, 223, 301] outbreak studies, surveillance [127, 131, 352], literature reviews [172, 175,

282, 312, 329], population-based studies [336, 352] and simulation modeling [275]. Standard testing of food-handlers during outbreaks [115], and education of food-handlers [131] were demonstrated to be effective in preventing foodborne viral disease. Genotyping can help provide evidence for the source of outbreaks [127]. The risk of HAV infections is substantial because of waning population immunity in industrialized countries [204, 354]. Prevention through vaccination is recommended for food handlers [22]. The alternative approach, post-exposure prophylaxis, is not feasible in the context of (hidden) foodborne transmission unless a potential contamination event has been identified early, and the size of the exposed group is clearly defined [2]. Not allowing ill food-handlers back to work until 48–72 h after their recovery is recommended. Compliance to this measure reportedly can be increased by the following personnel management options: absence of fear of job-loss when reporting illness [295], paid sick leave [131], education of food-handlers [131, 160], and willingness among managers to pay higher wages for trained workers [228].

Conclusion

Despite the fact that the surveillance of foodborne viral disease is minimal, significant progress has been made in our understanding of the epidemiology of foodborne viral infections, stressing that their relevance is not likely to diminish in the near future. Current regulations need to be reconsidered, as they are based on bacterial pathogens and do not suffice for control of viruses. Clear data gaps have been identified that should be the focus of future research and eventually provide the evidence for prevention and intervention strategies.

Medline Search

1. Food-Contamination, Food-Poisoning or Food-Microbiology or Gastroenteritis
2. Virus-Diseases, Viruses or Norovirus or Caliciviridae or Caliciviridae-Infections
3. Rotavirus or Rotavirus-Infections or Hepatitis-A or Hepatitis-A-virus
4. Hepatitis-E or Hepatitis-E-virus
5. (nonbacterial or non-bacterial or norovirus* or Norwalk or rotavirus or hepatitis) in ti,ab
6. #1 and (#2 or #3 or #4 or #5)
7. ((food-borne or foodborne) and (viral or virus*)) in ti,ab
8. viral contamination in ti,ab
9. Food-Handling or Food-Services or Zoonoses
10. Ruminants or swine or Poultry or Meat or Dairy-Products
11. (handler* or handling or animal origin or animal human or zoono*) in ti,ab
12. (hotel* or restaurant* or cafetaria* or ship*) in ti,ab
13. (meat or poultry of fish or shellfish) in ti,ab
14. (#6 or #7 or #8) and (#9 or #10 or #11 or #12 or #13)

Review Methodology: An extensive Medline search was done using search items Food-Contamination, Food-Poisoning, Food-Microbiology, food or Gastroenteritis in combination with any of the following terms in all subheadings: Virus-Diseases, Viruses or Norovirus or Caliciviridae or Caliciviridae-Infections, Rotavirus or Rotavirus-Infections or Hepatitis-A or Hepatitis-A-virus, Hepatitis-E or Hepatitis-E-virus, nonbacterial or norovirus* or Norwalk or rotavirus or hepatitis. Additional search was done to include references with any of the following keywords in title or abstract: foodborne or food-borne and viral or virus*, viral contamination, Food-Handling or Food-Services or Zoonoses, Ruminants or swine or Poultry or Meat or Dairy-Products, handler* or handling or animal origin or animal-human or zoono*, hotel* or restaurant* or cafetaria* or ship*, meat or poultry of fish or shellfish. The period covered was from 1 January 1997 to 15 May 2008. In addition, we used references from the articles obtained by this method to check for additional relevant material. We also spoke to colleagues and checked for manuscripts not yet published. The main focus of this review was on the latest developments on food-borne viruses [172].

General Discussion

Chapter 10

As described in chapter 1 of this thesis, public health institutes like Municipal Health Services (GGD'en) and the Food and Consumer Safety Authority (nVWA) in the Netherlands are obliged to undertake action when food forms a threat to public health. However, foodborne viral outbreaks, and especially the diffuse (international) ones, may be difficult to recognize as such. These are outbreaks that involve a batch of food that was exported to several countries, causing outbreaks in these countries simultaneously. These outbreaks are likely to occur when foods are involved that do not need further processing before consumption, like oysters or raspberries, and that are contaminated early in the food supply chain. In this thesis, the following five methods were described which may facilitate the recognition of diffuse foodborne outbreaks:

- In chapter 3 a surveillance system for outbreaks of gastroenteritis onboard cruise ships is proposed as a method to monitor the emergence of new variants and the background prevalence of strains in the population
- In chapter 5 a web-based tool is presented which can be used to select the outbreaks suspicious of foodborne norovirus transmission from those likely to be caused by person-to-person transmission.
- In chapter 6, the use of genotype profiles is proposed as a method to separate foodborne outbreaks caused by food contaminated early in the food supply chain from those contaminated at the end of the food supply chain by food handlers.
- In chapter 7 an algorithm is described which can be used to identify potential international foodborne norovirus outbreak events.
- In chapter 8, a generic method is described to identify the informative genomic region of a pathogen, with norovirus as an example. This method may be useful for laboratory networks to evaluate their efforts made in identifying foodborne outbreak events.

These methods may facilitate in pinpointing a single food product as the source of infection. Important challenges for that are recognizing the involved transmission route, and linking outbreaks caused by rapidly changing virus strains. With the implementation and acceptation of new methods combining epidemiological and molecular data, the epidemiological evidence for association with a foodborne source may become sufficient evidence for undertaking international action. Here we discuss the possibility of identifying international foodborne norovirus outbreaks, the current impact of such outbreaks, and how the work presented in this thesis may contribute to minimizing foodborne viral illness in the near future.

The Basics for Identifying International Foodborne Outbreaks

Tackling the Tangling of Transmission Modes

A complication in recognition of foodborne outbreaks is the tangling of transmission modes, with person-to-person transmission quickly taking over, which may mask initial introduction of the virus via food and make the identification of a point source challenging (chapters 3 and 4) [342]. This problem may be solved by separating early from late cases

during risk factor analysis [116, 351], as was also the approach in the investigation described in chapter 4. In this outbreak investigation, the short incubation period of noroviruses together with the presence of a menu facilitated the identification of a potential link to food. Nevertheless, a link to food could not be confirmed onboard this specific ship, despite the confirmed presence of norovirus in raspberries; raspberries were not in the menu as a separate dish and were thus not included in the questionnaire. Moreover, the raspberries may have been contaminated by an infected food handler, as the sample was derived from an opened bag. The causal relationship was even more difficult to confirm since a newly emerging variant, II.4-2006b, was involved which was commonly seen at that time (chapter 3). Moreover, the GII.4 genotype is relatively more frequently seen in person-to-person outbreaks (chapter 5). Thus, even when food is microbiologically suspected, and a thorough epidemiological investigation is performed, a link to food may be difficult to confirm. This was also seen during one year of intensified outbreak surveillance [336]. Considering this knowledge, underreporting of foodborne outbreaks reported can be considered likely, as was also concluded previously [246].

Surveillance to Fill the Knowledge Gap of the Background Prevalence of Strains

Despite the short incubation period of norovirus, which is an advantage that facilitates source tracing, other characteristics of noroviruses – like their variability – complicate the identification of a common foodborne source with an international distribution. The interpretation of potential links should be done with caution. For reliable sequence clustering in order to identify links between cases and food, and for the use of molecular information for identification of common source events, knowledge about the background diversity of the viruses in the population under investigation is important. The interpretation of finding closely related strains was an important problem encountered during the emergence of cruise-ship related outbreaks in 2006, described in chapter 3. The similarity in the setting of these outbreaks was suspicious of a common foodborne source, for example through a common ship supplier, or a frozen food product commonly used on ships, or shellfish like oysters that are commonly consumed during luxurious cruises. Nevertheless, thorough analysis of the background strain population circulating among humans at that time indicated that the most probable explanation for these outbreaks was increased norovirus activity in the community, which coincided with the emergence of 2 new GGII.4 variant strains, as could be recognized by the use of polymerase region A detection which allowed for international comparison [338]. Still, full capsid sequencing was needed as an additional step in order to recognize these strains as new variant strains. If these outbreaks were not compared to the background prevalence of strains at that specific time, these cruise-related outbreaks would have been interpreted as linked common source outbreaks, and the global emergence of the GII.4-2006a and GII.4-2006b variants might have been wrongly attributed to a common source event.

Obtaining insight in the background prevalence of viruses thus provides the possibility to distinguish outbreak situations from epidemic situations in endemic regions. This can be accomplished by surveillance or monitoring with ongoing reporting of laboratory and epidemiological findings.

Although norovirus surveillance systems are present for humans and for shellfish, surveillance of foodborne gastro-intestinal disease is mainly focusing on bacteria like *Salmonella* spp, STEC and *Campylobacter*. Despite earlier recommendations [172], systematic surveillance for foodborne viral disease other than NoV outbreaks [174] has not yet been achieved, or – where present – has to deal with considerable underreporting [236].

A Consensus Target Enabling Reliable Phylogenetical Analysis

With the increasing variability of norovirus strains, the need for a consensus region for international typing and comparison of strains is of increasing concern. Reliably reconstructing the genetic distances between international outbreaks strains is dependent on well aligned sequences. This is currently challenging with the different lengths and target regions of the genome sequenced in the different laboratories. Currently, various RT-PCR methods are used in different countries [204, 348]. To identify viral outbreaks with a common foodborne source, the future focus should be on data-exchange between laboratories and countries according to an agreed protocol [225] (chapter 8). Despite the preference for comparison of whole genomes in linking international outbreaks and the rapid developments in this field [140], it is not likely that its application will become common use in diagnostics in the near future. Obtaining a full genome sequence of pathogens that cannot be cultured will remain a challenge for regional diagnostic laboratories [51, 178, 234]. Therefore, the method described in chapter 8 is likely to contribute to standardization in the years to come. The approach showed that the various commonly applied PCR regions may not allow for distinction of international outbreak events from the background diversity of strains. This was best illustrated with the outbreak originating from Lourdes that involved people diagnosed in multiple countries using different PCR targets [342]. On the other hand, like cruise ships, Lourdes is a place where many people visit and may introduce new strains. This is especially true for common strains like the GII.4-2006b at that time in 2008, as well as during the cruise-related outbreaks in 2006 [341]. The inconclusiveness is a large disadvantage of retrospective analysis, and confirmation in prospective studies are planned to be performed, to confirm the informative region for linking outbreaks (Sukhrie, unpublished work).

Awareness of Mixtures of Viruses Indicating Sewage Contaminated Foods

Another situation for special attention is in the case of sewage contaminated foods, i.e. early in the food supply chain. Sewage contaminated foods are a public health risk, since multiple strains may be present on foods contaminated by this route, coinciding with the risk of recombination and development of novel strains after simultaneous ingestion [14, 292]. Recent molecular data have shown that mixtures of viruses appear fairly common in shellfish [198] and sewage contaminated foods [110]. Unfortunately, phylogenetic analysis does not assist in identifying multiple strain outbreaks and focusing on closely related strains only may result in missing international common source events. However, the analysis of identical strain clusters illustrated that multiple strain clusters showed other characteristics like association with foodborne transmission or involving genotypes with a foodborne preference, and were still picked up in the algorithm of chapter 7 to identify international outbreak events [344]. Moreover, as each of these strains is still likely to evolve within an outbreak, phylogenetics may still add to the identification of sewage contaminated foods causing outbreaks.

Impact of International Foodborne Norovirus Outbreaks

The Estimated Proportion of Foodborne Illness

In recent years, noroviruses have been increasingly recognized as important causes of foodborne disease. However, the under-reporting, the lack of surveillance systems and the inability of existing systems to determine the proportion of disease that is transmitted by foodborne routes relative to other common routes make it difficult to estimate the proportion of viral illness that is foodborne. In a prospective study of one year of intensified outbreak

surveillance in the Netherlands in 2002, 21% of all gastroenteritis outbreaks were found to be foodborne, of which 25% were caused by noroviruses. An expert elicitation resulted in 17% (95%CI 16-47) of all norovirus outbreaks to be caused by foodborne transmission in the Netherlands [130]. The work in this thesis importantly contributed to more precise estimates of the contribution of the different ways food can be contaminated. For the first time estimates could be given for the proportion of foodborne outbreaks due to contamination early in the food supply chain. Retrospective analysis of all norovirus outbreaks in Europe between 1999 and 2008, as described in chapters 5 and 6, estimated that 21-22% of all reported norovirus outbreaks were caused by foodborne transmission, while 16-18% had been labeled as such. A total of 9% of all reported outbreaks were estimated to be common source outbreaks, of which 80% were estimated to be *international* common source outbreaks (chapter 7). These estimates were only possible owing to the existence of a systematically collected outbreak dataset containing combined virological and epidemiological data over a prolonged period of time. Such datasets are rare, but should be the future focus not only for noroviruses but also other foodborne pathogens.

International Studies of the Burden of Norovirus Illness

Although estimates for the foodborne proportion are available, most research focuses on estimating the proportion of human illness to be attributed to norovirus. Such estimates commonly rely on costly population-based studies designed for other gastroenteritis and foodborne pathogens as well. Few of such studies were performed in the United States, Australia, the United Kingdom and the Netherlands. They estimated the burden of viral gastroenteritis between 1.3 and 31 infections per 1,000 inhabitants [8, 73, 126, 229]. These findings suggest differences in exposure between countries, but this may be the result of different methods used, or different seasons covered in the studies. Norovirus infections show a seasonal pattern with high prevalence in winter but occur all year round [18] which may be especially true for foodborne outbreaks. Moreover, noroviruses may be omnipresent without being the cause of illness, which is complicating the burden estimates. The age-adjusted seroprevalence of asymptomatic norovirus infection was found to be 12% in England [263], whereas norovirus was detected in 5.2% of the healthy controls in a Dutch population-based study [73].

The Burden of Norovirus Illness in the Netherlands

This population-based study in the Netherlands in 1999, SENSOR, is to date the basis for estimating the burden of norovirus illness in the Netherlands. This study resulted in ranking of pathogens, and estimates of burden, costs for society, and total amount of Disability Adjusted Life Years (DALYs) [74, 335]. The burden of norovirus was estimated to be 450 DALYs with an estimated incidence of 470,000 cases in 1999 (2.9% of the Dutch population) and costs for society were estimated to add up to 25 million euros in 2004 [162]. The SENSOR study was performed over a decade ago, among sporadic cases only, and did not include norovirus outbreaks. Moreover, for outbreaks the epidemiology may be different [38] with consequences for burden estimates. At the time of the Sensor study, norovirus infection was considered a mild and self-limiting disease. Over the past decade, significant progress has been made in the field of norovirus research, yielding new knowledge about the virus and its consequences. For example, recent studies revealed that norovirus infections are potentially life-threatening for the elderly and that significant mortality may be associated with norovirus infections [20, 281]. Newly emerging variants have been recognized every two years since 2002, causing epidemics across Europe and worldwide [1, 182, 205, 308, 341]. A recent

investigation accounting for these new insights estimated that again 3% of the Dutch population in 2009 experienced a norovirus infection, but that the incorporation of the new insights with respect to mortality of the disease in burden estimates resulted a higher amount of DALYs, i.e. 1760 (Verhoef et al. unpublished data) compared to the previously estimated 450 [335].

Tools for Recognition of International Foodborne Outbreaks Provided by this Thesis

Surveillance to Fill the Knowledge Gap of the Background Prevalence of Strains

Because outbreaks on cruise ships are likely to occur when new variants circulate, and new variants are likely to cause an epidemic norovirus seasons, an active reporting system for outbreaks of gastroenteritis onboard cruise ships could function as an early warning system. If such a system would be implemented, public health authorities can be warned in summer and prepare for an epidemic winter season. Although the recognition of foodborne outbreaks may not be improved by such a surveillance system, it will be helpful to separate a common source event from the emergence of new variants. Both situations ask for different measures from public health authorities.

Tackling the Tangling of Transmission Modes

The methods described in chapters 5, 6 and 7 can be applied to cut down the total number of outbreaks to those for which foodborne proportion is probable (chapter 5, selection tool). This information can be useful in source tracing activities and may add to increasing the success rate of such actions. A next step would be to cut down these probable foodborne outbreaks to those foodborne outbreaks due to contamination early in the food supply chain (chapter 6, genotype profiles), and limit the in-depth investigations to those that are likely to be common source foodborne outbreaks, while separating the national from international ones (chapter 7, algorithm). Whereas the genotype profiles are applicable to a surveillance dataset in a retrospective way, the selection tool and the algorithm can be used prospectively for newly reported outbreaks. The algorithm to identify potential foodborne outbreak events [344] can alarm a user when entering a strain that may be linked to other outbreaks suspected to be related to food. Although the algorithm is not validated for prospective use, the selection tool is. These three methods are planned to be incorporated into the web-based database of the FBVE network, currently known as the Molecular Platform (www.rivm.nl/pubmpf/norovirus/database). The Molecular Platform is an initiative to develop a generic database structure for reporting of sequence data accompanied with some basic epidemiological data, and to develop typing tools facilitating communication between laboratories.

A Consensus Target Enabling Reliable Phylogenetic Analysis

The method described in chapter 8 can be incorporated in the Molecular Platform, and run on request as an evaluation point to guide laboratory efforts in recognizing international outbreaks once a large enough dataset of reference sequences of adequate length is available. The method may be applicable to other pathogens for which full length sequences together with epidemiological information are available. Although the costs of whole genome sequencing are decreasing, obtaining a whole genome sequence of pathogens that cannot be cultured will remain out of reach for daily practice in regional diagnostic laboratories. Therefore, focusing on shorter consensus fragments sufficiently sensitive in recognizing

outbreak events is likely to improve the reliability of sequence comparison and thereby the identification of foodborne outbreak events.

Future Prospectives

The new estimates for foodborne outbreaks with distinction between foods contaminated early or late in the food supply chain were only possible owing to the existence of a systematically collected outbreak dataset containing combined virological and epidemiological data over a prolonged period of time. Such datasets are rare, but should be the way forward not only for noroviruses but also other foodborne pathogens. To identify viral outbreaks with a common foodborne source, the future focus should be data-exchange between laboratories and countries according to an agreed algorithm [225] (chapter 8). Although not all regional laboratories may be able to perform sequencing and molecular typing of viruses, this situation is improving rapidly, especially with the development of multiplex RT-PCR methods that can detect several viruses, or several genotypes of a virus, in one run [283]. Unfortunately, typing is commonly not considered of relevance for diagnostic purposes, as specific treatment is not available, and symptomatic treatment does not depend on the genotype involved. Therefore, public health institutes need to be made aware of the applicability of molecular typing in their decision making with respect to source tracing, so that they can request this extra effort from the laboratories and thereby contribute to secondary prevention of illness.

The time may come that data-collection is no longer the limiting factor in gaining knowledge. The restraints are in the computational possibilities, given the calculation time for thorough analysis of the rapidly growing sequence databases. If public health institutions want to learn from their precious data, there is definitely a need to invest in improvement of computational possibilities. Since other research fields, like computational biology, have made considerate progress on this topic already, the most efficient way will be to collaborate with existing bioinformatics institutions. On the other hand, other fields like bacteriology and parasitology may very well profit from the progress made in the field of virology. Since viruses have small genomes, the development of generic methods may be possible while requiring relatively limited calculation time and storage capacity.

The work described here may become even more useful in the future, as foodborne viral outbreaks due to food contamination early in the food supply chain will continue to occur. With the current developments in the field of climate changes and limited resources, water reuse is increasingly recognized as a solution for water scarcity [114, 219]. However, this may form a risk when used as irrigation water. Moreover, the use of bacterial indicators for fecal contamination may not suffice for viruses, and these may still be present after treatment [188], and contamination of fresh produce can be considered likely [261].

Overall Conclusion

In all, significant progress has been made in the use of molecular typing of noroviruses for food safety perspectives. Although data gaps have been identified that should be the focus of future research, some gaps are filled. Evidence for prevention and intervention by food safety authorities may need to be derived from new methods combining epidemiological and molecular data, of which some examples are presented in this thesis. To enable development

of such methods for other foodborne pathogens, systematic collection of combined molecular and epidemiological data should be the way forward not only for noroviruses but also other foodborne pathogens. Given the development towards globalization, and the reuse of wastewater for irrigation, the burden of foodborne viral illness is not likely to diminish. The added value of molecular typing needs to be communicated towards routine diagnostic laboratories, since typing results are currently mainly used and interpreted in the scientific community. The food-industry and the scientific community need to work together in a joint effort to develop an integrated plan of action to address foodborne viral infections. International collaborations combined with rapid exchange of outbreak data are crucial to identify viral outbreaks with a common foodborne source.

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Acknowledgments

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Acknowledgments

The work described in chapter 2 was supported by the European Commission in three projects: (i) FBVE (Foodborne Viruses in Europe), rapid detection of transnational foodborne viral infections and elucidation of transmission routes through molecular tracing and development of a common database, contract no. QLK1-1999-00594; (ii) DIVINE, prevention of emerging enteric viral infections: diagnoses, viability testing, networking and epidemiology, contract no. 2003213; and (iii) EVENT (Enteric Virus Emergence, New Tools), providing tools to prevent emergence of enteric viruses, contract no. 502571.

With respect to the work described in chapter 3, we thank the Center for Infectious Disease Control in Iceland and the Royal Infirmary of Edinburgh in Scotland for sending us their laboratory test results. We also thank staff at the Essex Health Protection Unit, the Lothian National Health Service board in Edinburgh, the Municipal Health Service Nijmegen, and Municipal Health Service Rotterdam for data sharing and their kind cooperation. We are grateful to Hendriek Boshuizen for assistance in the statistical analyses. This work was supported by the Dutch Food and Consumer Product Safety Authority, the European Commission DG Research Quality of Life Program, 6th Framework (EVENT, SP22- CT-2004-502571), and SG SANCO (DIVINE-net, 2003213).

With respect to the work described in chapter 4, we are grateful to the involved cruise ship's crew, for their approval for this study and their kind cooperation in data-collection. We thank Evelyn Depoortere for initiating this investigation and René Koenen for giving us the opportunity to perform the study. We would also like to thank Yvonne van Duynhoven and Annelies Kroneman for their support during the preparation of the study on short notice. We would also like to express our gratitude for the contribution of the following: Michel Heijneman, who took environmental, food and water samples during the onsite investigation; Nathalie te Loeke, who was involved in analyzing all environmental swab and food samples for the presence of norovirus; Bas van der Veer, who performed laboratory testing of all stool samples; Willemijn Lodder for the analysis of water samples and ice cubes; Soizick LeGuyader, Leena Maunula and Froukje Lodder-Verschoor who analyzed the raspberries for presence of norovirus.

We thank Stef Bronzwaer for allowing us to use the draft EFSA guidelines for testing our surveillance database for the analysis described in chapter 5, the epidemiologists and data-entry personnel participating in the FBVE network for their interviews about the surveillance of norovirus infections, Amal Chatterjee for editorial assistance, and the anonymous reviewers for their useful suggestions. This work was supported by the Dutch Food and Consumer Product Safety Authority, the European Commission DG Research Quality of Life Program, 6th Framework (EVENT, SP22-CT-2004-502571) and SG SANCO (DIVINE-net, 2003213).

We thank Annelies Kroneman, Soizick LeGuyader, and Leena Maunula for critically reviewing the work described in chapter 6 and Amal Chatterjee for editorial assistance. This work was supported by the Dutch Food and Consumer Product Safety Authority, the European Commission Directorate General Research Quality of Life Program, 6th Framework (EVENT, SP22-CT-2004-502571) and Secretariat General SANCO (DIVINE-net, 2003213).

We thank Erwin Duizer and Leena Maunula for critically reviewing the work described in chapter 7, Lucy Phillips for editorial assistance, and the Dutch Food and Consumer Product Safety Authority and the European Commission for financial support.

We thank Adam Meijer, Ingeborg Boxman and Marijn van Ballegooijen for helpful discussions that helped us to improve the work described in chapter 8.

We are grateful to Erwin Duizer, Saskia Rutjes and Sanela Svraka for their helpful discussions on the topics described in chapter 9.

Persons contributing and countries represented in the FBVE network: H. Vennema, E. Duizer; D. Brown, B. Adak, J. Gray, J. Harris, M. Iturriza; K.-H. von Bonsdorff, L. Maunula, M. Kuusi ; B. Böttiger, K. Mølbak, G. Falkenhorst, C. Johnsen; K.-O. Hedlund, Y. Andersson, M. Thorhagen, M. Lysén, M. Hjertqvist; P. Pothier, E. Kohli, K. Balay, J. Kaplon, G. Belliot, S. LeGuyader; A. Bosch, A. Dominguez, J. Buesa, A. Sanchez Fauquier, G. Hernández-Pezzi; G. Szücs, G. Reuter, K. Krisztalovics; M. Poljsak-Prijatelj, D. Barlic- Maganja, A. Hocevar Grom; F. Ruggeri, I. Di Bartolo; E. Schreier, K. Stark, J. Koch, M. Höhne; M. Lynch, B. Foley, P. McKeown, S. Coughlan; K. Vainio, T. Bruun.

Samenvatting

Dankwoord

Samenvatting

Norovirussen zijn een van de belangrijkste veroorzakers van uitbraken en sporadische gevallen van gastro-enteritis in Nederland, maar ook wereldwijd. De symptomen bestaan meestal uit projectiel braken en/of waterdunne diarree, waarvan men na enkele dagen hersteld is. Een norovirusinfectie kan echter een ernstiger verloop hebben in kwetsbare personen, zoals ouderen en immuun-gecompromitteerden. Persoon-op-persoon transmissie komt het meeste voor. Toch wordt norovirus vaker via voedsel overgedragen dan enkele jaren geleden werd gedacht. Na introductie van het virus via besmet voedsel, kan persoon-op-persoon transmissie zorgen voor verdere verspreiding. Een voedselgerelateerde uitbraak kan daarnaast niet alleen een lokaal, maar ook een internationaal probleem zijn. De toenemende globalisering van de voedselmarkt heeft tot gevolg dat een voedselpartij in verschillende landen aangeboden wordt voor consumptie. Daardoor zal het steeds vaker voorkomen dat norovirus uitbraken geografisch verspreid, oftewel diffuus, plaatsvinden na de consumptie van besmet voedsel. Men moet dan vooral denken aan voedselproducten die vroeg in de voedselketen besmet worden door contact met fecaal vervuild water en vervolgens geconsumeerd worden zonder verdere 'virusdodende' bewerkingen, zoals koken. Norovirus besmette oesters en frambozen zijn daarvan voorbeelden. Tot op de dag van vandaag is het erg lastig om virussen in of op voedsel aan te tonen. Vaststellen dat meerdere norovirusuitbraken een gemeenschappelijke voedselbron hebben is om deze reden moeilijk. In dit proefschrift worden nieuwe methoden beschreven, die kunnen helpen bij het herkennen van (internationale) diffuse voedselgerelateerde norovirusuitbraken. Er is gekozen voor een aanpak waarbij zowel epidemiologische als virologische gegevens worden gebruikt.

De basis voor dit proefschrift zijn gegevens uit een surveillance databank, waarin sinds 1999 zowel epidemiologische als virologische gegevens van norovirus uitbraken in Europa geregistreerd worden. Deze registratie wordt gedaan door 13 deelnemende landen van het Foodborne Viruses in Europe (FBVE) netwerk en de databank wordt dan ook de FBVE databank genoemd. In **hoofdstuk 2** wordt de databank beschreven. Uit deze beschrijving blijkt dat er grote verschillen tussen landen zijn in wijze van surveillance. Zo zijn niet alle landen in staat om epidemiologische èn virologische gegevens op gecombineerde wijze te registreren. Verder zijn bepaalde landen meer gericht op het herkennen van voedselgerelateerde uitbraken, terwijl andere landen zich vooral richten op uitbraken in bepaalde settings, zoals ziekenhuizen. Uit de analyse van de databank gegevens blijkt dat norovirussen van het genotype II.4 het meeste voorkomen tijdens uitbraken en dat het aantal uitbraken per jaar vooral hoog is wanneer er een nieuwe variant van dit genotype opduikt. Norovirusuitbraken vinden vooral plaats in de winter. Bij de opkomst van een nieuwe variant is al in het voorjaar of de zomer een toename in het aantal uitbraken te zien. Dat werd bijvoorbeeld zichtbaar door de toename van het aantal norovirus uitbraken op cruiseschepen in het voorjaar van 2006, zoals beschreven in **hoofdstuk 3**. In korte tijd werden 43 norovirus uitbraken op 13 schepen gemeld. In een dergelijke situatie kan gedacht worden aan een gemeenschappelijke (voedsel)bron, bijvoorbeeld wanneer de schepen gebruik maken van eenzelfde voedselleverancier. Dit laatste bleek hier niet het geval. In deze periode is verder epidemiologisch onderzoek verricht aan boord van een van deze cruiseschepen. Zoals beschreven is in **hoofdstuk 4**, was het niet mogelijk de bron van het norovirus onomstotelijk aan te wijzen, ondanks dat norovirus aangetoond werd op frambozen afkomstig van een reeds aangebroken verpakking. Geconstateerd werd dat kennis van de diversiteit aan norovirustypen bij uitbraken in de populatie ('achtergrond diversiteit') vastgelegd in de FBVE databank van groot belang is geweest in de periode van de cruiseschip

gerelateerde uitbraken. Door deze specifieke kennis kon en kunnen toekomstige internationale uitbraken of nieuw opduikende varianten herkend worden als een bijzondere situatie die afwijkend is van de achtergrond diversiteit. Een surveillance systeem voor uitbraken van gastro-enteritis op cruiseschepen zou daarnaast ook een mooie mogelijkheid bieden voor het verkrijgen van gegevens, waarmee al in de zomer een idee kan worden gekregen over de aard van de norovirus activiteit in de daarop volgende winter. Dat maakt het op tijd nemen van voorzorgsmaatregelen mogelijk. Een andere belangrijke conclusie was dat er indicatoren nodig zijn voor het herkennen van norovirusuitbraken die zeer waarschijnlijk geassocieerd zijn met besmet voedsel, mogelijk gevolgd door transmissie van persoon-op-persoon of via de omgeving.

Voor het bepalen van bovengenoemde indicatoren is de FBVE databank gebruikt en zijn de daarin opgenomen gegevens geanalyseerd. Dit heeft onder andere de in **hoofdstuk 5** beschreven selectie tool opgeleverd. De selectie tool kan door medewerkers in het veld van de publieke gezondheid gebruikt worden wanneer er een norovirus uitbraak bij hen gemeld wordt en waarbij men zich moet afvragen of besmet voedsel een mogelijke bron was. In het piekseizoen van norovirus is het voor de GGD nagenoeg onmogelijk iedere gemelde uitbraak even grondig te onderzoeken om de bron of transmissie route te identificeren. Met de tool kan op systematische wijze een selectie gemaakt worden van uitbraken waarbij klachtonderzoek of bronopsporing naar besmet voedsel nuttig is èn er dus een verhoogde kans is op het aantonen van een relatie met voedsel. Geschat wordt dat na gebruik van de selectie tool het aantal uitbraken waarvoor nog serieus gekeken moet worden naar de rol van voedsel daalt tot eenderde van het totaal aan gemelde uitbraken. Op deze wijze kan efficiënt voldaan kan worden aan de wettelijke verplichting voedselgerelateerde uitbraken te herkennen en te melden. Geconcludeerd werd dat norovirussen *niet* behorend tot het genotype II.4 relatief vaker voorkomen bij voedselgerelateerde uitbraken. Wanneer we gedetailleerder naar de onderverdeling in genotypen kijken, zoals in **hoofdstuk 6** is gedaan, dan blijkt dat bepaalde niet-II.4 genotypen inderdaad vaker voorkomen bij vastgestelde voedselgerelateerde uitbraken. Hiermee kon genotype profiel worden opgemaakt, waarmee onderscheid gemaakt kon worden tussen verschillende soorten voedseluitbraken, namelijk die veroorzaakt door voedsel dat al vroeg in de voedselketen besmet is geraakt en uitbraken waarbij voedsel vlak voor consumptie besmet werd, bijvoorbeeld door een geïnfecteerde voedselbereider. Daardoor kon voor het eerst een schatting gemaakt worden van het percentage uitbraken als gevolg van besmetting van voedsel tijdens de productiefase; deze werd geschat op 21%. Daarnaast werd geschat dat bij 25% van de uitbraken, waarbij tijdens de registratie in de FBVE databank aangegeven was dat een voedselbereider de bron was, het voedsel al mogelijk eerder in de voedselketen besmet was geraakt.

Met het onderscheiden van norovirus uitbraken door enerzijds voedsel besmet tijdens de primaire fase of anderzijds voedsel besmet aan het einde van de keten door de voedselbereider, is de eerste stap gezet in de richting van herkennen van diffuse (internationale) uitbraken. Om een beter inzicht te verkrijgen in de eigenschappen van het bovengenoemde type uitbraken, is een gedetailleerde analyse uitgevoerd met de epidemiologische gegevens behorend bij geregistreerde uitbraken met identieke norovirus sequenties (**hoofdstuk 7)**. Dit heeft een algoritme opgeleverd waarmee, stap voor stap, mogelijke diffuse, internationale voedselgerelateerde uitbraken herkend kunnen worden. Op basis van deze analyse werd geschat dat 7% van alle gerapporteerde uitbraken in de FBVE databank mogelijk onderdeel was van een dergelijke internationale gebeurtenis. Dit is opmerkelijk omdat nog geen 1% als zodanig bekend stond. Een van de kenmerken van het

ontwikkelde algoritme is dat het gebaseerd is op identieke sequenties en daarmee ‘streng’ is. Het is mogelijk dat het genoom van de norovirussen in de tijd tijdens internationale uitbraken muteert, zeker in de meer variabele delen van het genoom, en sequenties niet meer volledig identiek blijven. Mogelijk is het gevonden percentage dus een onderschatting van het percentage van uitbraken dat onderdeel uitmaakt van een internationale uitbraak. Verder bleken sequenties van theoretisch dezelfde (internationale) uitbraak in de praktijk lastig te vergelijken. Onderzoeksgroepen hadden niet altijd hetzelfde gedeelte van het norovirus genoom gekarakteriseerd door middel van sequentie analyse. Wanneer men het bijvoorbeeld vergelijkt met een ‘vingerafdruk’ ter identificatie van een persoon, dan heeft men in het ene geval de vingerafdruk van de duim en in het andere geval die van de pink, waarbij je het terug moet zien te traceren naar één persoon. Bekend is dat niet alle gedeelten van het norovirus genoom even stabiel of variabel zijn. De vraag rees dan ook of het gedeelte van het genoom dát gebruikt werd voor het matchen van uitbraken wel de beste keuze was. In andere woorden in welke mate draagt een specifiek gedeelte van het genoom bij aan de identificatie van sequenties die behoren tot mogelijk dezelfde uitbraak ten opzichte van andere gedeelten van het genoom. Dit vraagt om een fylogenetische analyse aanpak, waarmee we met deze FBVE databank van grote omvang op het vakgebied van de bio-informatica terecht komen, zoals het onderzoek beschreven in **hoofdstuk 8**. Op basis van fylogenetische analyse van alle volledige norovirus capsides die ter beschikking stonden, is een methode ontwikkeld waarmee vastgesteld kan worden met welk gedeelte van het genoom het mogelijk is om vooraf gedefinieerde groepen correct te clusteren. De vooraf gedefinieerde groepen reflecteerden verschillende niveaus van resolutie, zoals die bekend zijn in de FBVE databank, namelijk het genotype niveau, het genotype II.4-variant-niveau en het uitbraak niveau. Het geïdentificeerde gedeelte van het genoom dat in staat bleek om deze niveaus correct te clusteren bevond zich in het variabele deel, wat gedeeltelijk overlapte met het P2 domein. Het behaalde resultaat kan echter gebiased zijn, omdat de methode nu is toegepast op een selectie van sequenties. Het is denkbaar dat bepaalde norovirussen interessanter zijn voor onderzoek en daarmee eerder in aanmerking komen voor het verkrijgen van een volledige sequentie van het capside. Mogelijk weerspiegelt de selectie daarom niet de volledige achtergrond diversiteit van sequenties in de populatie. Om daadwerkelijk inzicht te krijgen in het deel van het genoom dat het meest geschikt is voor het matchen van uitbraken, zal deze methode opnieuw toegepast moeten worden op een volledigere dataset. Dit kan bijvoorbeeld bereikt worden door tijdens uitbraken prospectief voor alle patiënten volledige capsides te sequensen en deze vervolgens te vergelijken met sequenties die aselect gekozen zijn voor volledig sequensen. De in **hoofdstuk 8** beschreven methode is een generieke methode en is daarom niet alleen voor norovirussen toepasbaar, maar is ook toepasbaar voor andere ziekteverwekkers waarvoor vergelijkbare databases aanwezig zijn. Daarnaast kan de methode ook gebruikt worden als een evaluatietool voor andere laboratoriumnetwerken om te bepalen of het door hen gekozen gedeelte van het genoom in staat is internationale voedselgerelateerde uitbraken op te sporen.

Samenvattend zijn in dit proefschrift de volgende vijf methoden beschreven en/of aanbevolen:

i) een voorstel voor surveillance naar uitbraken van gastro-enteritis op cruiseschepen om nieuwe varianten vroeg te onderkennen;

ii) een selectie tool gericht op de herkenning van mogelijke voedselgerelateerde uitbraken uit alle gemelde uitbraken;

iii) norovirus genotypenprofielen voor herkenning van contaminatie van voedsel vroeg in de voedselketen (primaire productie);

iv) een algoritme voor de herkenning van mogelijke internationale uitbraken met een gemeenschappelijke voedselbron;

v) een generieke methode om het gedeelte van het genoom van een ziekteverwekker (pathogeen) vast te stellen waarmee (epidemiologische) relaties tussen sequenties gelegd kunnen worden.

Wij denken dat de hier beschreven methoden kunnen bijdragen aan een verbeterde surveillance van voedselgerelateerde norovirus uitbraken. Zoals blijkt uit de diversiteit aan uitbraakrapportages in het literatuuroverzicht beschreven in **hoofdstuk 9** zullen voedselgerelateerde uitbraken door virussen in het algemeen en norovirussen in het bijzonder voorlopig niet ophouden te bestaan. Het onderzoek beschreven in dit proefschrift draagt bij aan de kennis over norovirus uitbraken door voedsel en de herkenning daarvan. De resultaten kunnen bijdragen aan de gerichte bestrijding van norovirus infecties via voedsel. Surveillance in de bevolking blijft van belang om inzicht te houden in de achtergrond diversiteit van virussen in de populatie, zodat voedseluitbraken tegen deze achtergrond diversiteit als zodanig zijn te herkennen.

Dankwoord

Yes, hij is af! Wat een heerlijk gevoel. Ik kan het nu wel bekennen: toen ik in juni 2006 bij LIS-VIR begon, wist ik niet echt het verschil tussen een virus en een bacterie. Echter, dat ik mij als een soort detective vast kon bijten in bronopsporing wist ik al wel door mijn *Legionella*-verleden. Ik ben aan dit promotietraject begonnen als epidemioloog en geëindigd in een identiteitscrisis: ben ik nu lab of epi? Een ding is me duidelijk geworden: het lab kan blijkbaar, net als statistiek voor sommigen, als een 'black box' fungeren. Je stopt er een epidemioloog in en er komt wat anders uit. Dat is voor mij een transformatieproces geweest, waarin ik ontzettend veel heb geleerd. En het mag duidelijk zijn, aan dit proefschrift hebben velen al dan niet bewust bijgedragen, en mijn dank daarvoor aan hen allen is groot.

Marion, als geen ander kan jij mensen inspireren. Door daarbij veel te vragen haal je het beste uit mensen. Zo had ik vooraf nooit gedacht dat ik uiteindelijk kon kiezen welke stukken ik wel, en welke niet, in mijn proefschrift wilde opnemen. Ik vind het geweldig zoals jij mij de mogelijkheden hebt gegeven mij te ontwikkelen. De ervaring in Australië was daarbij voor mij onvergetelijk. Ik heb daar mogen ervaren hoe het voelt om als expert op een vakgebied gezien te worden. Dit hoop ik nog vaker mee te mogen maken. En vooral hoop ik dat ik nog lang met je mag werken.

Wilfrid, je bent een man met een hart van goud! Ondanks al je tegenslagen afgelopen jaar, vond jij steeds de tijd om aan mijn 'deadlines' te voldoen. Met genoegen denk ik terug aan de leuke discussies die we hadden als kamergenoten op mijn EPI-dinsdag. Ik bewonder je creatieve vermogen om analyses steeds weer beter en leuker te maken. Ook met jou hoop ik nog lang samen te werken.

De heren Prof.dr. J.H. Richardus, Prof.dr. A.D.M.E. Osterhaus, Prof.dr. R.A.M. Fouchier, Prof.dr. R.A. Coutinho, Prof.dr. A.H. Havelaar, en Prof.dr. C.A.B. Boucher wil ik bedanken voor de bereidheid plaats te nemen in mijn promotiecommissie en voor de tijd en moeite die ze hebben genomen om mijn proefschrift te beoordelen.

Ingeborg, je hebt het vast al gezien: je hebt zelfs inspraak gehad op de titel. Je hebt waarschijnlijk meer dan je zelf door hebt invloed gehad op mijn promotietraject. Jouw kennis van virussen in voedsel is enorm groot en ik ben blij dat ik daar wat van mee heb kunnen krijgen. Ook Benno wil ik bedanken. Hij heeft deze samenwerking tussen nVWA en RIVM mogelijk gemaakt. Ik hoop dat de komende bezuinigingen ruimte laten voor deze mooie nVWA projecten.

Binnen het RIVM zijn er uiteraard veel mensen die een bijdrage hebben geleverd aan mijn promotietraject en proefschrift, zowel inhoudelijk als voor de ontspanning. Harry, Erwin en Annelies, jullie deur stond altijd voor me open en wat ben ik vaak jullie kamer binnengelopen. Het was steeds weer een verrassing voor wie ik nu weer kwam. Misschien dat ik niet altijd even blij reageerde als een paper weer aangepast moest worden, maar ik vond jullie suggesties iedere keer verbeterend en ik heb ontzettend veel van jullie geleerd. En Bas, dankzij jou was het voor mij mogelijk de norovirus-data te analyseren. Verder mijn kamergenoten Mariken, Adam en Janko, en recenter Harrie en Annemarie, waarmee ik lief en leed heb gedeeld. En dan natuurlijk de aio's en aio-achtigen, in volgorde van binnenkomst Joukje, Sanela, Marcel, Era, Faizel, Sabine vd S, Dominique, en EUPHEMs Sabine D en Giovanna. Hoewel ik nooit op de aio-kamer heb gezeten, kon ik altijd bij jullie binnenlopen voor gezelligheid en een luisterend oor. We slepen elkaar er wel doorheen! Natuurlijk ook de collega's bij EPI, en dan vooral de

gastrogroep en de hardloopgroep met ‘aanvoerder’ Liselotte. Ik vind het heel gezellig (en uiteraard ook nuttig ☺) om de dinsdagen met jullie door te brengen. Marc, unfortunately She didn’t Appear in this thesis… I really enjoyed working with you on our Lourdes study, which still is my absolute favorite. I am curious where you will end up eventually. Ook de intervisiegroep wil ik bedanken voor hun verhelderende blik op de werksituaties. Verder Theo, Fatima, Nel en Carola van het secretariaat, en de mensen van Beheer, heel erg bedankt voor jullie persoonlijke betrokkenheid en het uit handen nemen van allerlei regeldingen!

Special thanks to all co-authors of papers included in this thesis, to members of the FBVE network, and to Kelly Williams and Bruno Sobral and their colleagues at the VBI department of Virginia Tech. It was a great opportunity to call upon your expertise and you all have enormously contributed to the final contents of this thesis by inspiring presentations, discussions and comments.

Verder wil ik graag al mijn vrienden bedanken voor de gezellige en broodnodige ontspanning in de vorm van etentjes, weekendjes weg, relaxen in sauna’s, shoppen. Iedereen bedankt voor jullie relativerende vermogen dat soms nodig was om mij als vakidioot en workaholic af te remmen. En hoewel ik niet iedereen bij naam zal noemen, wil ik wel graag specifiek Irene&Jurjen, Heleen&Robert en Marieke&Hans bedanken. We kennen elkaar al gemiddeld 19 jaar (range 7-31, mediaan 20), en wat hebben we in die jaren veel meegemaakt samen. Jullie onvoorwaardelijke vriendschap is van ongekende orde. Ik wou dat ik 3 paranimfen mocht hebben… en ik wens iedereen zulke vrienden toe als jullie! En natuurlijk Froukje, zonder jou had ik Rob misschien wel nooit ontmoet! Ook de ‘kookclub’, al sinds 1991 eten we met regelmaat bij elkaar, wat een tijd. Dit jaar ons vierde lustrum en ik zou zeggen: vooral doorgaan!

En dan mijn lieve familie. Vol vertrouwen al die jaren, ook al was het proefschrift wel erg lang ‘bijna af’. Lieve mama, je bent een geweldige, lieve, zorgzame moeder die altijd voor ons klaar stond en staat. Ik ben net als jij heel blij dat ik deze datum heb kunnen kiezen. Helaas is het papa niet gegund dit mee te maken, maar gelukkig heb ik hem nog wel kunnen vertellen dat ik ga promoveren, en toen al was hij trots. Lieve papa, deze had je nog van me tegoed! En je bewaarwijn is nu vereeuwigd. Lieve Ruud en Rob, mijn grote broers en grote voorbeelden. Ook al zijn we nog zo verschillend, ik heb van kleins af aan heel veel aan jullie gehad. De Marsupilami-song (hoeba hoeba hoeba hap hap hap) is jullie vergeven hoor! Ook de rest van de familie en mijn schoonfamilie hebben met hun geduld en vertrouwen bijgedragen aan het bereiken van deze dag, en ik ben heel blij dat ik deze dag met jullie allemaal mag vieren.

Lieve Rob, ‘mijn’ Rob, wat ben ik blij dat ik jou jaren geleden op Schiphol tegen het lijf liep. Geweldig zoals wij elkaar aanvullen en telkens weer met grote beslissingen op één lijn liggen. Onze levensweg gaat wat buiten de gebaande paden, en dat gaat ons goed af. Een dag niet gelachen is een dag niet geleefd, dat is een uitspraak die op ons gebaseerd moet zijn. Lieverd, jouw onuitputtelijke bron van steun heeft mij nog het meeste gegeven bij het afronden van dit proefschrift. Het lijkt me fantastisch om volgend jaar weer met jou over de Chinese muur te lopen!

Het afgelopen jaar heb ik mijn vrienden en familie veel te weinig gezien. Aan jullie allemaal: kom snel weer eens (virusvrij) eten!

Curriculum Vitae

Publicaties

PhD Portfolio

Curriculum Vitae

Linda Verhoef werd geboren op 15 augustus 1973 in Arnhem. Het VWO diploma behaalde zij in 1992 aan het Thomas à Kempis College in Arnhem. Na een jaar Economie aan de Universiteit van Amsterdam, besloot zij Voedingskunde en Diëtetiek te gaan studeren aan de Hogeschool van Amsterdam. Zij deed hier onderzoek in Bangladesh naar de acceptatie van sojabonen als vleesvervanger, en liep stage bij UMC St. Radboud als diëtiste, waarna zij in 1997 afstudeerde. Vervolgens is zij Voeding en Gezondheid gaan studeren aan de Universiteit Wageningen, waar zij in januari 2000 afstudeerde in de richting Epidemiologie. In een gecombineerd voedingskundig en epidemiologisch afstudeervak deed zij onderzoek bij de Soedanese bevolking naar genetische polymorfismen die een rol spelen bij het ontstaan van leverkanker na consumptie van aflatoxinen in pinda's. In 2000 werkte zij aan de Universiteit Wageningen als toegevoegd docent bij de vakgroep Wiskunde, in 2001 als statistisch onderzoeker bij het Centraal Bureau voor de Statistiek, in 2002 als milieu-epidemioloog bij het RIVM, en van 2003 tot 2006 bij de Hulpverleningsdienst Kennemerland als epidemioloog bij het nationale projectteam BronopsporingsEenheid *Legionella*-pneumonie (BEL-project).

In juni 2006 trad Linda in dienst bij de afdeling Virologie van het Centrum Infectieziektebestrijding van het RIVM. Vanaf dat moment hield zij zich bezig met uitbraakonderzoek en onderzoek naar de haalbaarheid van een detectiesysteem voor voedselgerelateerde uitbraken door (noro)virussen, onder begeleiding van Prof.dr. Marion P.G. Koopmans en Dr. W. van Pelt. Dit heeft geresulteerd in diverse publicaties, waarvan een aantal de basis vormden voor dit proefschrift.

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Submitted

1. Boxman ILA, **Verhoef L**, Dijkman R, Hägele G, Loeke te N, Koopmans M. A year round prevalence study for the environmental presence of norovirus in catering companies without a recently reported outbreak of gastroenteritis. Submitted.

2. **Verhoef L**, Williams KP, Kroneman A, Sobral B, van Pelt W, and Koopmans M, on behalf of the Foodborne Viruses in Europe Network. Data-driven selection of the informative genomic region for linking outbreaks with norovirus as an example. Submitted.

3. Heijnen J, Rondy M, **Verhoef L**, Teunis P. The role of health care workers on transmission of norovirus during an outbreak: a network analysis. Submitted.

Erasmus MC
Universitair Medisch Centrum Rotterdam

PhD Portfolio Summary

Summary of PhD training and teaching activities

Name PhD student: Linda Verhoef Erasmus MC Department: Research School: Molecular Medicine Postgraduate school	PhD period: June 2006 – June 2010 Promotor: Prof.dr. Koopmans Co-promotor: Dr. van Pelt

1. PhD training

	Year	Workload in Hours
General academic skills		
- English coaching taalcentrum VU	2008	40
- Scientific writing	2006	80
Research skills		
- Beginning Perl for Bioinformatics	2009	8
- Bionumerics introduction course	2009	16
- R introduction and advanced course	2007	80
- Attribution course MedVetNet Work Package 28	2006	16
- SAS regression techniques	2006	40
- SPSS Maps		
In-depth courses (e.g. Research school, Medical Training)		
- 15th international workshop on virus evolution and molecular epidemiology	2009	40
- Advanced principles of infectious disease epidemiology	2007	40
- Lab for Epi EAN/LIS	2008	8
Presentations		
- Oral presentation Australian Food Safety Conference, Melbourne	2010	8
- Oral presentation Food and Waterborne Disease network, Dublin.	2010	4
- Oral presentation Southwestern University, Dallas, Texas	2010	2
- Oral presentation VBI Virginia Tech, Blacksburg, Virginia	2010	8
- Poster 15th international workshop on virus evolution and molecular epidemiology, Rotterdam, the Netherlands	2009	5
- Master Class with Mark Pallanch (CDC Atlanta), Bilthoven, the Netherlands	2009	1
- Poster ESCAIDE, Stockholm, Sweden	2007	10
- Poster EPIET, Menorca, Spain	2006	10
- Oral presentation DIVINE/EVENT network meeting, Rome	2006	8

- Oral presentation EVENT network meeting, Uppsala	2009	6
- Oral presentation CIb PhD meeting, Zandvoort	2009	2
- Oral presentations (8) for department LIS-VIR	2006- 2010	40
- Oral presentations (4) for visitors of department	2008-2010	20
International conferences		
- Australian Food Safety Conference, Melbourne	2010	24
- Annual meeting FWD Network ECDC, Dublin	2010	16
- EVENT network meeting, Uppsala	2009	16
- Final conference of the SHIPSAN project, Athene	2008	24
- Future challenges to microbial food safety, Wolfheze	2008	40
- ESCAIDE, Stockholm, Sweden	2007	40
- EPIET, Menorca, Spain	2006	40
Seminars and workshops		
- DIVINE/EVENT network meeting, Rome	2006	40
Didactic skills		
- Presentation course	2006	40
- English Lingurama course	2009-2010	30
Other		
- rapporteur WHO expert meeting on viruses in food, Bilthoven	2007	40
2. Teaching activities		
	Year	**Workload in hours**
Lecturing		
- Erasmus Winter Course, simulation study	2011	30
- Erasmus Summer Course, lecture Detection of international viral foodborne outbreaks.	2010	8
- Training course outbreak investigation in Indonesia	2008	80
Supervising practicals and excursions		
- Erasmus Winter Course	2010	8
- ECDC training course outbreak investigation in Europe	2008-2009	100
- EPIET module lab-epi, Bilthoven	2009	20
Other		
- Supervising EPIET fellow in outbreak investigation and writing manuscript	2009	100
- organizing symposia on noroviruses	2008, 2011	60

Notes: