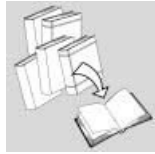


REVIEW



'Norwalk-like viruses' as a cause of foodborne disease outbreaks

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SUMMARY

While outbreaks of foodborne disease remain an important public health concern, their aetiology is not identified in a majority of instances. In targeted studies, the application of newly developed molecular assays has demonstrated that a large proportion of these outbreaks may be caused by the 'Norwalk-like viruses' (NLV), a genus of genetically related viruses belonging to the family *Caliciviridae*. NLV outbreaks associated with consumption of faecally contaminated oysters are frequently reported and can best be controlled by preventing contamination of oyster-harvesting waters. Infectious foodhandlers are another frequent source of contamination, and such transmission can be minimised by exclusion of ill foodhandlers and the maintenance of strict personal hygiene. Molecular assays have greatly refined the epidemiological investigation of foodborne NLV outbreaks, allowing the linking of outbreaks in different locations and permitting the identification of the virus in the implicated vehicle. The development of simpler and more sensitive assays and their use on a broader scale will assist in defining the true burden of foodborne NLV outbreaks and improve strategies for their prevention and control. Copyright © 2001 John Wiley & Sons, Ltd.

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INTRODUCTION

Despite major advances in preventive health over the last century, foodborne illnesses remain a widespread and growing global public health problem [1]. Each year, an estimated 1.5 billion diarrhoeal episodes, resulting in 2.5–3.2 million deaths in children <5 years of age, are reported worldwide [2,3]. The World Health Organization estimates that a substantial proportion of these diarrhoeal episodes may be associated with food contaminated by microbes. The burden of foodborne disease is well documented in some industrialised countries. For example, in the United States, foodborne pathogens cause an estimated 76 million illnesses, 325 000 hospitalisations and 5000 deaths each year [4]. While less

well documented, it is likely that the burden of foodborne illness is far greater in developing countries where levels of hygiene and sanitation are poorer. Most cases of foodborne disease are sporadic and are often not reported. Outbreaks represent only a small tip of the iceberg of foodborne illness but are a cause for great concern because of their potential to affect large numbers of people. A variety of agents — bacteria, viruses, parasites, toxins, metals and prions — cause foodborne illness, which can range from mild diarrhoea and vomiting to life-threatening syndromes with multi-organ involvement. In the past 20 years, many newly identified pathogens (e.g. *Escherichia coli* O157:H7, *Cyclospora cayentanensis*) have been recognised as causes of foodborne disease. However, even the most recently available data from industrialized countries indicate that an aetiological agent is not identified in most outbreaks of foodborne disease [5]. Among the main obstacles in identifying the causative agent are the failure to collect appropriate clinical specimens in a timely manner and the unavailability of simple and sensitive diagnostic tests for

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Abbreviations used:

IAHA, immune adherence haemagglutination assay; NLV, Norwalk-like virus; SLV, Sapporo-like virus; SRSV, small round-structured virus; VLP, virus-like particle

some agents. Consequently, pathogens that are difficult to detect or for which assays are not readily available are under-represented in the list of known causes of foodborne outbreaks.

The 'Norwalk-like viruses' (NLVs), previously called the small round-structured viruses (SRSVs) because of their morphological appearance, are clearly one of the under-recognised causes of outbreaks of foodborne disease. The prototype agent of this genus of viruses, Norwalk virus, was first identified in 1972 in stool samples from volunteers fed faecal filtrates from elementary school students who were affected by an outbreak of gastroenteritis in Norwalk, Ohio, in 1968 [6,7]. Progress in the research on NLVs in the two decades following this discovery was slow because of the inability to adapt the virus to grow in cell culture, the unavailability of a useful animal model for disease and the lack of simple and sensitive diagnostic assays. In the past decade, however, breakthroughs in determining the molecular biology of NLVs [8–11] and the subsequent development of molecular diagnostic methods [12–26] and their application to clinical and epidemiological studies have greatly improved our understanding of the role of these viruses as causes of gastroenteritis in humans. In this article, we present an overview of the role of NLVs in the aetiology of foodborne disease outbreaks.

THE 1970S AND 1980S — BEFORE THE AVAILABILITY OF MOLECULAR DIAGNOSTICS

In the two decades following its discovery, the detection of Norwalk virus in clinical specimens relied on electron microscopy (EM), immune (IEM), solid-phase radioimmunoassays (RIA), immune adherence haemagglutination assays (IAHA) and enzyme immune assays (EIA) [6,27,28]. Direct EM and IEM had relatively low sensitivity and required skilled microscopists and expensive equipment. The reagents for immunologic assays were based on clinical samples, with stools of infected persons providing antigen and convalescent-phase sera providing specific antibodies. Consequently, methods for detection of Norwalk virus were available in a limited number of research laboratories and were not suitable for large clinical and epidemiologic studies.

Nevertheless, early studies demonstrated the importance of NLVs as a cause of outbreaks of

gastroenteritis. For example, in 1979, Greenberg *et al.* found serologic evidence of Norwalk virus infection in 32% of 25 separate outbreaks of nonbacterial gastrointestinal illnesses examined [29]. Similarly, an aetiological role for NLVs was demonstrated in 42% of 74 outbreaks of acute nonbacterial gastroenteritis investigated by the Centers for Disease Control (CDC) from 1976 to 1980 [30] and in 82% of 34 non-bacterial gastroenteritis outbreaks that occurred in Tokyo during February 1985 to June 1991 [31]. By reviewing features of confirmed outbreaks of NLV gastroenteritis, Kaplan *et al.* developed clinical and epidemiologic criteria predictive of a Norwalk virus aetiology [32]. These included: (1) stool cultures negative for bacterial pathogens; (2) mean (or median) duration of illness 12–60 h; (3) vomiting in greater than or equal to 50% of cases; and (4) if known, mean (or median) incubation period of 24–48 h. Of 642 outbreaks of acute gastroenteritis reported to the CDC between 1975 and 1980, they showed that 23% of waterborne outbreaks, 4% of foodborne outbreaks, and 67%, 60% and 28% of outbreaks in nursing homes, in summer camps and on cruise ships, respectively, satisfied the criteria for Norwalk-like pattern [32].

The potential for foodborne transmission of NLV disease was first recognised following a large outbreak of gastroenteritis in Australia in June and July 1978 that affected at least 2000 persons [33]. The food implicated in this outbreak, oysters, as well as other shellfish were subsequently recognised as common vehicles of NLV infection and were implicated in several outbreaks worldwide [34–38]. In some outbreaks [36], the detection of Norwalk virus by RIA in clam and oyster specimens confirmed their epidemiologic link with disease. Investigations of these outbreaks and laboratory studies also identified several other features of shellfish-associated NLV disease that had important implications for prevention: (1) the implicated shellfish were often consumed raw [33–35]; (2) sewage contamination of the oyster-harvesting areas frequently preceded the outbreak [33,37]; (3) persons in widespread geographical areas could be affected because of the distribution of contaminated shellfish [33]; and (4) depuration (practice of holding oysters in tanks of disinfected water for a period of time) was not always effective in ensuring the safety of shellfish for consumption [39].

Besides shellfish, outbreaks associated with other food items were also reported [40–55]. Interestingly, in all except one of these outbreaks [40], cold food items involving bulk preparation such as salad [41,45–47,48,54], sandwiches [42,52], frosting [43] and cold meats [44] were implicated as the vehicles of infection. In the single outbreak where hot food items were implicated [40], as well as several other outbreaks associated with cold foods [42–44,47,49,50,53], contamination by an infectious food handler was suspected. Often, a foodhandler reported illness prior to, or while, preparing food items, but a foodhandler who was incubating the illness was suspected as the source of infection in one outbreak [42] and evidence for transmission from a foodhandler after recovery from illness was presented in two other outbreaks [47,50]. Iversen and colleagues suggested that long-term excretion of Norwalk virus by a symptom-free foodhandler was responsible for two outbreaks of gastroenteritis occurring 3 weeks apart [48], but the evidence supporting this claim was challenged by others [55]. A single outbreak was associated with contaminated celery that was exposed to non-potable water [54].

THE 1990S — THE MOLECULAR ERA

A breakthrough occurred in the early 1990s with the cloning and sequencing of the Norwalk virus [8] and Southampton virus [11] genomic RNAs. Knowledge of the genetic organisation of NLVs allowed the development of sensitive detection methods based on reverse transcription-polymerase chain reaction (RT-PCR) amplification and nucleotide sequencing of RT-PCR products provided an opportunity to study the similarity of viral strains [12,17]. When expressed in a recombinant baculovirus vector, the single capsid protein of Norwalk virus self-assembles into virus-like particles (VLPs) that have provided important tools for studying the immunology, structure, and, to some extent, the replication cycle of NLVs [20,22,24–26]. Expressed VLPs from Norwalk and several other NLV strains have been used as antigens to measure serum antibody responses to NLV infection [20,22,24–26,56,57] and as immunogens to generate hyperimmune animal sera.

The widespread availability and application of these molecular assays has clearly demonstrated that NLVs are a leading cause of outbreaks of foodborne gastroenteritis worldwide.

For example, NLVs were detected by RT-PCR assays in approximately 90% of 348 outbreaks of non-bacterial gastroenteritis that occurred between January 1996 and November 2000 in the United States and for which specimens were sent for testing to the CDC in Atlanta; of the outbreaks with available data, 39% were transmitted by food and 39% occurred in restaurants (Figures 1A and 1B) [58, CDC unpublished data]. Similarly, in England and Wales, NLVs accounted for more than one-third of all outbreaks of gastroenteritis reported between 1992 and 1995 [59]; foodborne transmission was documented in 14% of all NLV

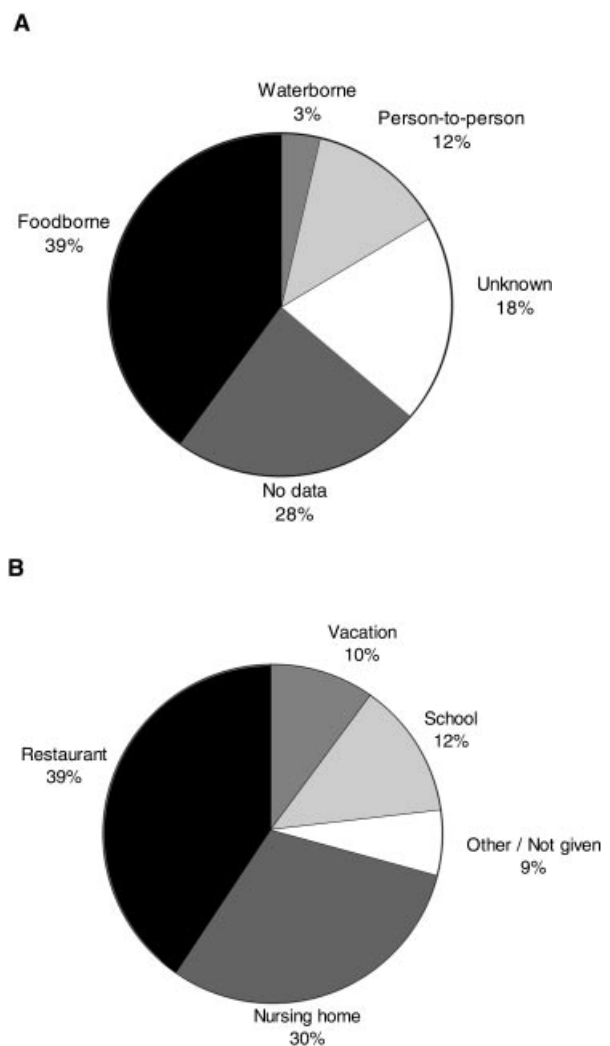


Figure 1. Mode of transmission (A) and settings (B) of 348 outbreaks of gastroenteritis reported to the CDC between January 1996 and November 2000

outbreaks. In the Netherlands, of the 184 outbreaks of suspected viral gastroenteritis that occurred between 1991 and 1998, 82% were confirmed by laboratory testing to be caused by NLVs [60]; 77% of outbreaks labelled as foodborne were caused by NLVs. In Japan, NLVs accounted for 68% of 387 outbreaks of non-bacterial gastroenteritis reported between January 1997 and March 1999 [61]; foodborne transmission was documented in more than two-thirds of outbreaks.

Molecular assays have also greatly refined the investigation of outbreaks of foodborne viral gastroenteritis. Assays have been developed to detect NLVs directly from shellfish [62–69], and their value is well illustrated by the investigation in 1993 of 23 clusters of acute gastroenteritis affecting several hundred people in six different US states [70,71]. In this investigation, the detection of viral genome with identical sequence in a 123-bp region from stools of patients in different states as well as the detection of a similar sequence in the implicated oysters confirmed the epidemiologic link between the cases and the source of the outbreak [70–72]. Molecular assays have also been used to study the kinetics of NLV accumulation in shellfish and the effect of depuration on the reduction of NLV titres in tissues of shellfish [73], as well as to demonstrate that seasonal physiological changes undergone by oysters affect their ability to accumulate viral particles from estuarine waters [74].

During the 1970s and the 1980s, the association of food items with illness in NLV outbreaks relied primarily on demonstration of an epidemiologic association and, sometimes, the detection of bacterial indicators of faecal contamination in the implicated food item. Methods were developed in the late 1990s to detect NLV contamination of other food items besides shellfish [63,75,76]. The utility of these assays is well illustrated by a recent outbreak that affected students at a university in Texas [77]. Illness in this outbreak was epidemiologically linked with eating sandwiches at the university's main cafeteria deli bar, and the RT-PCR analysis of samples of the deli meat detected NLV genome with the same sequence in the capsid region as that of virus from stools of the ill university students, confirming that the sandwiches were the vehicle of infection.

The application of molecular diagnostics has also better defined the role of foodhandlers in NLV

outbreaks. Previously, foodhandlers were linked as the source of an outbreak through circumstantial evidence of illness [40,42–44,47,49,50,53] and, sometimes, evidence of infection [48,50]. With molecular assays, infection in foodhandlers can be detected with greater sensitivity and their association with illness can be further evaluated by comparing the nucleotide sequence of the viral genome obtained from specimens of foodhandlers and the persons affected by the outbreak [77,78]. The greater sensitivity of these assays has also provided new insights into the infectiousness of foodhandlers. Previously, based on the findings of epidemiologic investigations and a study that examined by IEM shedding in volunteers challenged with Norwalk virus [79], it was believed that foodhandlers were infectious during and up to 48–72 h after recovery from illness. In a more recent outbreak investigation [78], viral genome was detected by RT-PCR in two foodhandlers, one of whom was free from disease and another sick foodhandler whose specimen was obtained 10 days after resolution of illness. These patterns of shedding are consistent with those observed in more recent studies of human volunteers that used recombinant-antigen based EIAs for viral detection [80,81].

DISCUSSION

In the past decade, the development and application of molecular diagnostics for NLVs and the consequent understanding of the viral genome has considerably improved our understanding of the biology and epidemiology of these viruses. Sequence and genetic analysis has shown that the NLVs, previously called SRSVs, are indeed genetically different from the other genus of caliciviruses that infect humans, 'Sapporo-like viruses' (SLVs), which were previously distinguished from NLVs based on the observation of typical cup-shaped depressions on the virion. Nucleotide sequence analysis has further allowed the classification of NLVs into three genetic groups — genogroup I, genogroup II and genogroup III — and phylogenetic classification has defined clusters within genogroups. The antigenic diversity of NLVs has also been recognised and this diversity in general has correlated with phylogenetic clusters [82]. Understanding these complex antigenic and genetic relationships between NLVs has allowed constant refinement

of molecular assays, but these assays still have many limitations.

Antigen-detection EIAs for NLVs are quite specific and in studies of outbreaks of gastroenteritis in different populations, the overall detection rates using these assays [19,83,84] were substantially lower than those using RT-PCR [58,60,85,86]. Antibody detection EIAs are more broadly reactive and perform well when the outbreak strain is genetically identical to the strain used in the assay [56,82], but heterologous antibody responses are difficult to interpret. The occurrence of dual infections of NLVs from different genetic groups and natural viral recombinants further complicates the diagnostic picture [87–89]. Recognition of a greater number of NLV strains, improved understanding of the genetic relationship among these strains, expression of more recombinant proteins representative of the different genetic types, and the detection of common epitopes shared by several strains of NLVs should allow further improvement of these assays. The utility of these assays in particular settings could be further improved by monitoring the prevalent genetic types of NLVs, which may assist in the selection of an appropriate panel of EIAs.

RT-PCR assays provide a sensitive and broadly reactive tool to detect NLV genome in faecal specimens, and, in the absence of commercial EIAs, have been widely used for the detection of NLVs in clinical and environmental specimens. However, no universal primers to detect all NLVs are available. Primers based on the most conserved polymerase region of the NLV genome have been widely used worldwide, and the results of these studies indicate that it may not be possible to develop universal primers that can detect all the different genotypes of NLVs. Alternative approaches, such as developing several sets of primers that detect the polymerase region of different NLVs, degenerate primers, or primers from another regions of the genome such as capsid primers, may be required to broadly detect all strains of NLVs.

While the application of RT-PCR assays to detect NLV genome in food, water and other environmental specimens [62–68,63,75,76,90–97] provides opportunities to study patterns of transmission and implicate vehicles of infection during outbreak investigations, the low concentrations of

virus and the presence of inhibitory substances in these specimens often requires the concentration and purification of virus prior to amplification assays. The efficiency of such assays can also be improved by first identifying the NLV strain from stools of patients and designing strain-specific RT-PCR primers. At the present, the relative insensitivity of RT-PCR methods for detection of virus in food and environmental methods allows only the interpretation of positive results and precludes their routine application in foodborne NLV outbreak investigations and use for food quality monitoring.

The improvement of diagnostic methods will not only enhance our understanding of the role of NLVs in foodborne disease outbreaks, but will also assist in developing strategies for prevention and control. While shellfish are clearly recognised as a common vehicle for NLV infection, currently available laboratory assays cannot be readily used for monitoring contamination of either the waters in shellfish-harvesting areas or the shellfish supplies. Until such assays are developed or better indicators of viral contamination become available, measures to avoid contamination of waters in shellfish-harvesting areas (e.g. surveillance of shorelines to identify possible sites of contamination of water, prohibiting overboard dumping of faecal wastes from boats) remain the most effective means of preventing outbreaks. It is likely that thorough cooking will also reduce the risk of illness, but outbreaks have been associated with cooked shellfish [98,99], and the stability of NLVs to heat is presently difficult to study because human volunteer studies are required to study the viability of the virus. The adaptation of NLVs to growth in cell culture will allow simpler laboratory studies and remains a high priority.

While foodhandlers clearly play an important role in the aetiology of NLV outbreaks, several important issues concerning NLV transmission by foodhandlers remain unclear. In the 1970s and 1980s, based primarily on epidemiologic data, the hypotheses that foodhandlers may transmit virus with asymptomatic infections, before the onset of clinical disease, or for prolonged periods after recovery were presented [42,47,48,50] and were sometimes challenged [55]. These hypotheses have been supported by data from more recent molecular studies showing that volunteers challenged with NLVs can shed viral antigen even in the

absence of symptoms and that viral antigen can be recovered in stool for more than a week after recovery from clinical illness [80,81]; however, it is not known whether the detected antigen is simply soluble viral antigen or the entire infectious virus. Similarly, while outbreak investigations have implicated as the source of infection food-handlers without clinical disease or before the onset of clinical symptoms [78], it is difficult to definitively exclude in these circumstances other unrecognised sources of contamination or inadequate recall on the part of the food-handler. Careful epidemiologic and laboratory studies are needed to determine the duration a foodhandler remains infectious. The development of quantitative assays will allow assessment of the timing of maximal viral shedding, which will help determine the period during which a foodhandler is most infectious.

CONCLUSIONS

The application of molecular diagnostics for detection of NLVs has clearly demonstrated that these viruses are among the leading causes of outbreaks of non-bacterial gastroenteritis worldwide. While these assays are being increasingly used worldwide, the development of simple, sensitive, specific and broadly reactive assays that can be routinely used in microbiology laboratories remains a high priority. Improving assays for detection of NLVs in environmental specimens and refining them to permit quantitative evaluation of viral contamination will be valuable for the prevention and control of foodborne NLV disease. Success in adapting NLVs to grow in cell culture will allow assessment of the viral replication cycle, immunologic correlates of protection against illness, and the stability of NLVs to temperature and disinfectants. The past decade has seen remarkable progress in research on NLVs which is likely to continue and accelerate in the coming years, leading to a better understanding of their role in the aetiology of foodborne disease outbreaks and improved approaches for prevention and control.

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