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THE DESCRIPTIVE EPIDEMIOLOGY OF NORWALK-LIKE VIRUSES

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ABSTRACT

THE DESCRIPTIVE EPIDEMIOLOGY OF NORWALK-LIKE VIRUSES

By

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Norwalk-like viruses are responsible for causing millions of cases of gastro-enteritis each year. They are ubiquitous and are highly infectious. Norwalk-like viruses cannot be cultivated outside of man, and prior to the development of novel molecular detection methods, the virus was difficult to detect in specimens. Norwalk-like viruses are commonly spread by several modes of transmission. The consumption of raw molluscan shellfish is a common mode of infection. Infected food handlers are responsible for transmitting the infection to large numbers of diners in food service establishments. Sewage-polluted water supplies have also been responsible for causing large-scale outbreaks. Most recently, hard epidemiological evidence for airborne spread via aerosols has been established. Recommendations for prevention include better monitoring of shellfish beds, using a viral indicator; improved food handler education regarding personal hygiene and the prohibition of bare-hand contact of ready to eat foods; more strict monitoring of well construction and sewage disposal systems, and meticulous environmental decontamination of spaces where potential cases have vomited. Improved surveillance will enhance our knowledge of the epidemiology of these viruses.

To my husband Joel and my daughters Chrissie and Jackie, whom I love.

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Without my friends and family, the writing of this review would have been much more difficult. During my work at Ingham Country Health Department, I have had the opportunity to work with many capable, energetic and dedicated public health professionals. While investigating an outbreak of foodborne illness which eventually was proven to be caused by Norwalk-like viruses, I decided to research further into the epidemiology of these viruses to answer the many questions which came to mind in the course of our work. The administration of Ingham County Health Department offered me unflagging moral and material support during my studies. I would like to thank F. Robert Godbold, Terry Anderson, and Bruce Bragg for their support and encouragement. I would also like to thank Wendy Little and Lora McAdams for their word process assistance which saved me countless hours of frustration. And thanks to my mentor and friend Walter Mack, for whom 20 years of exhortation has at last paid off. I would also like to thank my family, who have patiently endured and encouraged me during the researching and writing of papers, studying for midterms, and the other pressures which academic study can exert on an active family's life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CDC . . . . . Centers for Disease Control

ELISA . . . . . Enzyme Linked Immunosorbent Assay

EU . . . . . European Union

GI, GII . . . . . Genogroup I , II

HAV . . . . . Hepatitis A Virus

HACCP . . . . . .Hazard Analysis Critical Control Point

IEM . . . . . Immune Electron Microscopy

NLV . . . . . Norwalk-like Viruses

NSSP . . . . . National Shellfish Sanitation Program

NV . . . . . Norwalk Virus

PCR . . . . . Polymerase Chain Reaction

PFU . . . . . Plaque Forming Unit (s)

RIA . . . . . Radioimmunoassay

rNV VLP . . . . . Recombinant Norwalk Virus Virus-like Particles

RT-PCR . . . . . Reverse Transcription Polymerase Chain Reaction

TD . . . . . .Travelers' Diarrhea

INTRODUCTION

A viral etiology for outbreaks of acute infectious nonbacterial gastroenteritis was first confirmed in 1972 (89), when virus was isolated from an outbreak in Norwalk, Ohio. Christened Norwalk virus, it was subsequently shown to be a frequent cause of foodborne and waterborne gastroenteritis in the United States. During the 1970's and early 1980's, the clinical and epidemiological features of such outbreaks were characterized. An increasing number of different viral agents, with clinical aspects similar to that of Norwalk virus, were also identified during this time. This group became known as "small round-structured viruses" or Norwalk-like viruses (NLVs).

Laboratory identification of these viruses was hampered by the fact that the viruses do not grow in cell cultures or animal models, so confirmation of outbreaks was rare. Sero-epidemiology has confirmed that infection with NLV is extremely prevalent worldwide, and generally occurs at younger age in developing countries. Surveillance and reporting of NLV outbreaks have also been deficient, and the role of NLV illness in the United States and worldwide has probably been grossly underestimated. Recently developed laboratory methods have enabled confirmation of NLV etiology, and can be expected to confirm the suspected role of NLV as a major cause of infectious nonbacterial gastroenteritis worldwide. Public health professionals working "in the trenches" as health department sanitarians and disease control professionals may enhance their investigational abilities relative to gastroenteritis outbreaks by having a clear understanding of the clinical and epidemiologic features of Norwalk-like viruses. This literature review will hopefully enchance this understanding.

Chapter 1

DESCRIPTION OF NORWALK-LIKE VIRUSES

Norwalk virus (NV) is the prototype of the Norwalk-like group of viruses (NLVs). This group is also called "small round structured viruses" because viewed using electron microscopy they have an amorphous surface with a feathery ragged outline that lacks geometric symmetry. These 27-35 nm viruses also share other characteristics: they have similar densities in cesium chloride (1.34 to 1.42 g per milliliter), they cannot be cultivated in cell culture, and they derive from epidemics of gastroenteritis. It is estimated that an infectious dose of NLV may be as little as 10-100 virus particles. Many strains are named after the location of the outbreak. NLVs possess single stranded RNA of positive sense, and are members of the family Caliciviridae (3, 12, 84).

Chapter 2

CLINICAL FEATURES OF NLV INFECTION

Description of Symptoms

Outbreaks caused by Norwalk-like viruses are characterized by median, dose dependent incubation periods of 24-48 hours (range 2 hours to 7 days) , median duration of 12-60 hours, and a high percentage of patients with nausea, vomiting, cramps and diarrhea (9, 19, 43, 74, 91). Sudden projectile vomiting has been particularly associated with NLV illness without a defined prodrome (19, 21). Children and adolescents are likely to experience vomiting more frequently than diarrhea, while adults frequently experience higher rates of diarrhea than of vomiting (1, 94, 158, 169). Myalgia, sore throat and chills are less commonly reported (91). Headache (43%) and fever (25-49%) are also reported frequently (8, 35, 43). The disease is self-limiting and generally benign, occurring most frequently from September to March in the northern hemisphere. It seldom requires medical treatment and has no known sequelae (19).

Histopathological Features of NLV Infection

The histological response to NLV infection was characterized shortly after the infectious agent was characterized (2, 13, 147). Typically, studies involved the use of volunteers who ingested bacteria-free filtrates prepared after passage through a human agent. Baseline biopsies were performed, establishing that entirely normal tissues were present before the experimental infection with NLV. Pre-exposure biopsies showed that overall duodeno-jejunal cell architecture was normal at baseline, featuring tall villi and

short crypts, and a villus-height to crypt-height ratio of at least 3:1. There were no absorptive cell abnormalities, and polymorphonuclear leukocytes were absent (2, 147).

Post-exposure biopsies showed a consistent pathology in subjects who became ill. A mucosal lesion of the proximal small intestine was seen 48 hours after ingestion of the NLV agent. The virus replication appears to occur in the mucosal epithelium, resulting in a broadening and flattening of villi; the villus-height to crypt-height ratio was reduced to 2:1. Damage to mucosal epithelial cells resulted in crypt cell hyperplasia. Many polymorphonuclear leukocytes and increased numbers of mononuclear cells were seen throughout the lamina propria (2, 147).

In general, the epithelial absorptive cells were decreased in height and became disorganized. There were focal areas of epithelial cell vacuolization with non-lipid staining material. The epithelial cells showed a dilation of the rough and smooth endoplasmic reticulum, along with an increase in multivesiculate bodies. Increased numbers of lysosomal bodies were seen. In most cases, the microvilli were shortened, and intercellular spaces were widened and filled with an amorphous electron-dense material. Increased numbers of mononuclear cells and some polymorphonuclear leukocytes had also infiltrated the intercellular spaces between epithelial cells (2, 147). Direct evidence of viral maturation was lacking; no viral particles were observed when specimens were examined using electron microscopy (2, 20). In contrast to infection by invasive bacterial agents such as *Shigella* and enteropathogenic *E. coli*, the mucosa remained intact.

Rectal and colonic biopsies taken at the height of illness showed a normal histologic pattern. This evidence, along with the absence of fecal leukocytes in Norwalk-induced disease, suggests that the colonic mucosa is not involved in NLV syndrome (2,

12). Gastric lesions are not seen, and gastric secretion of hydrochloric acid, pepsin, and intrinsic factor are unaltered. Gastric emptying was markedly delayed, however, and this abnormal gastric motor function probably explains the frequent nausea and vomiting characteristic of NLV illness (12).

Peak viral shedding generally occurs 25-72 hours after inoculation, and has been shown to persist up to and beyond 7 days (57, 133). This is true of both symptomatic and asymptomatic infections: those with no symptoms have been shown to shed virus for up to 6 days after challenge (57).

By 48 hours after ingestion of NLV, two of four asymptomatic volunteers had developed mucosal lesions which were indistinguishable from those seen in the two volunteers with overt clinical illness. The histologic abnormalities were still present 5 days after ingestion of NLV, about two days after symptoms had cleared. However, though still present, the epithelial cell abnormalities and polymorphonuclear leukocyte infiltration were less severe (2, 147). Convalescence biopsies done two weeks after inoculation showed that the mucosa had returned to normal in all cases.

To study changes in enzymology, Agus et al (2) assayed brush border enzyme activities in post-inoculation and convalescence biopsy specimens, and compared changes experienced in each subject to that subject's own baseline information. They found that percent changes of alkaline phosphatase in ill volunteers (-49.3%) and well volunteers (+1.4%) were significant ($p<0.01$). Similarly, the percent change in trehalase activity at the time of illness (-61.%) was significantly different ($p=0.02$) from what was seen in well volunteers (-16.2%). Percent changes in sucrase and lactase levels were also decreased during illness, though the changes were not statistically significant (2). Transient

malabsorption of D-xylose and fat has also been demonstrated during acute NLV infection; the malabsorption is no longer present at 9-11 days after illness (2, 13, 147).

Several researchers have observed virus capsid protein antigens and soluble antigens in stools of patients infected with NV (57, 66). It appears that soluble antigen, which apparently is lacking in viral nucleic acid, is present in higher concentrations than capsid proteins. It is not known whether the soluble antigen is simply a degradation product whose appearance may be variable, or if it has a specific biologic function in viral replication (57).

Immune Response to NLV Infection

Studies of serological response to NVs using recombinant capsids indicate that infection results in a rise in serum IgG and IgA antibodies, with IgM being observed in the majority of volunteers (160). Several investigators have demonstrated that, paradoxically, volunteers challenged with NLV who became ill were more likely to have high, rather than low, prechallenge serum antibody titers (11, 57, 58, 73, 113, 160). Treanor et al (160) speciated the antibodies, showing that mean pre-challenge serum levels of IgG , IgA and IgM were higher in individuals who subsequently developed illness. Serum IgM responses were more common in ill individuals than in those who had subclinical infection, but IgM responses were also noted in those with asymptomatic NV infections. It has been suggested that IgM responses may be more strain-specific than IgG (83). It has also been shown that a wide distribution of antibody levels exists in the initial serum specimens from patients involved in outbreaks of gastroenteritis, from less than 2,000 to greater than 240,000 IgG units (122), and that the absence or presence of antibodies in the initial serum

did not correlate with protection against the occurrence of a subsequent seroconversion. These findings are consistent with the suggestion that serum antibody is not protective against NV infection (11, 58, 88, 138,160). It should be noted that conflicting results have been obtained in studies of naturally acquired NV infection in Panama and Bangladesh (10, 142). Interestingly, in a subject who was monitored continuously over two years, antibody levels declined rapidly to the pre-challenge level after one year (39).

Graham et al (57) described an association among infected groups wherein increases in antibody titers in convalescent sera were significantly higher in subjects who had vomiting or vomiting and diarrhea. The presence of vomiting and nausea, and headache or body aches correlated with the magnitude of the seroresponses. They also noted that titers of pre-existing antibody were significantly higher in subjects who excreted virus.

Parker et al (134) found that serum IgA response among volunteers was variable and unpredictable. One ill subject mounted a significant IgG response but serum IgA could not be detected in pre- or post-challenge sera. Two ill subjects who showed no significant IgG response showed a significant IgA response. One asymptomatic subject showed significant rises in both IgA and IgG titers. In summary, the mechanisms of seroresponse to NLV infection are poorly understood.

The secretory immune response to NLV infection is also complex and poorly understood. An early study evaluated the intestinal immune response to NV, measuring the blocking activity of duodenal fluid in 14 volunteers, using a method which did not distinguish between specific antibody classes (11). In this study, titers of duodenal IgA for ill and well persons were similar, and none of the volunteers developed a fourfold increase

in titers. Furthermore, all persons who had blocking activity in their duodenal fluids before NV challenge became ill after the challenge, while only 46% of those without blocking activity became ill after the challenge. Okhuysen et al (130) similarly demonstrated that the presence of high titers of specific anti-NV duodenal IgA in volunteers before challenge correlates with the likelihood of development of clinical illness and the failure of protection against subsequent challenge.

Parrino et al (138) observed that serum antibody titers to NLV were not protective against illness in a study of 12 volunteers repeatedly challenged with NV. The 6 who remained well did not experience gastroenteritis upon rechallenge. Four volunteers who became ill were challenged a third time 4-8 weeks after the second challenge. Only one became ill: this volunteer had a high antibody titer before the third challenge.

Parrino et al (138) also described a distinct group of individuals with low initial titers who were resistant to experimental infection even after three challenges. These individuals maintained low levels of serum antibody to NLV and failed to become infected. This finding has been confirmed by other researchers (57, 58, 88). It has been postulated that some subjects (identified in studies in the US) lacked a receptor that would allow NLV to enter the mucosal cells of the small intestine and were thus intrinsically nonsusceptible to NLV infection (88, 138). Whether specific immunologic, genetic, or other host factors cause this resistance is unclear. Volunteer studies conducted in the US using adults showed a lack of protective immunity, while apparent protective immunity has been reported among children in developing countries (10, 142). These children also had a history of rotavirus infection, perhaps of serial infections, possibly even concurrent with NV infection. The number and type of infections, and age at infection may play a role

in the development and retention of protective immunity against NLV. Temporary resistance to NLV may be passed to infants from mothers by breast milk containing virus-specific IgA (44, 125).

Some degree of acquired protective immunity has been observed. Parrino et al (138) observed a short-term immunity lasting up to 14 weeks. Such immunity was evidenced in 3 out of 4 volunteers in Parrino's study. Subjects were rechallenged with NV 28-42 months after the initial challenge, becoming ill both times. When challenged a third time 4 to 8 weeks later, they did not become ill. The fourth subject became ill after all three challenges. This apparent short-term immunity is consistent with other reports (13, 58). In a larger volunteer study, Gray et al (58) found that this apparently acquired short-term immunity lasted up to 6 months in most individuals. They suggested that acquired resistance to infection may depend on the rate and frequency of previous exposure of the individual to NV.

The lack of protection afforded by preexisting serum IgG and IgA antibodies (11, 58, 88), the unexplained, paradoxical association of high levels of serum antibodies with clinical illness after challenge, the development of short-term immunity (58, 138) and the occurrence of long-term immunity suggest that resistance to infection with NV may be mediated by cellular mechanisms, probably in the gastrointestinal tract (130).

Recent research toward vaccine production has used the recombinant Norwalk-virus-like particles (rNV VLP). Jiang et al (84, 87) discovered that the capsid protein of NV was one long molecule which could be produced in insect cells, and which self-assembles into a hollow but complete viral capsid. Ball et al (7) tested immunogenicity of this rNV VLP in mice, wherein IgG response arose by 9 days post-exposure, and IgA

response arose after 24 days. The rNV VLP is thus immunogenic, but as was shown in volunteer and outbreak-related serological studies, the induction of protective immunity appears to depend on cellular, host, and other factors which are not yet well understood.

Chapter 3

LABORATORY METHODS FOR IDENTIFICATION OF NLVs

The 27-nm Norwalk virus was identified by immune electron microscopy in 1972 in fecal material derived from a 1968 outbreak of gastroenteritis in Norwalk, Ohio. Classification and biochemical and molecular analysis of the NLVs have proven difficult because they could not be adapted for growth in tissue culture. Although NV induced subclinical infection in pygmy chimpanzees, all attempts to develop a practical laboratory animal model have been unsuccessful (62,164). Volunteer studies that were instrumental for the initial identification of this 27-nm virus were thus continued not only to gain an understanding of the natural history of NV and related viruses, but to generate fecal material containing NV for use as an antigen reagent.

Laboratory confirmation of the cause of outbreaks of food-borne and waterborne viral gastroenteritis requires either the detection of virus in stool or demonstration of a rise in specific antibody. Virus can be identified by detection of viral antigens, or visualization of the virus by electron microscopy, or by amplification of the viral RNA using PCR techniques. Proper collection and storage of specimens for testing, thus preserving the integrity of the viruses present in the specimen, is prerequisite for recovering evidence of viral infection. Instructions from the Centers for Disease Control and Prevention (CDC) for collecting specimens to evaluate outbreaks of viral gastroenteritis are summarized in Table 1 (31,74).

Table 1. Instructions for collecting specimens to examine for agents of viral gastroenteritis.

Parameter	Stool	Serum
Source	10 ill persons; 10 controls for comparison (optional)	Same persons from whom stool was collected (controls optional).
Specimen	At least 10 ml/person in clean dry containers	15 ml (adults) and 3 ml (children) blood specimens collected in tubes containing no anticoagulants.
Time	Within 48-72 hours after onset of illness	Collect acute phase specimens at same time as stool; collect convalescent-phase specimens 3-4 weeks after onset of illness.
Storage and Shipping	Immediately refrigerate at 4°C (39°F) Place bagged and sealed specimens with frozen refrigerant packs in insulated box. Send by overnight mail. DO NOT FREEZE.	Refrigerate tubes of serum until shipped with frozen refrigerant packs in insulated box. Keep specimens frozen by shipping on dry ice. Send by overnight mail.

Adapted from the CDC (31).

Immune Electron Microscopy (IEM)

Norwalk virus was first identified in filtrates of stools from people who were part of an outbreak of nonbacterial gastroenteritis in Norwalk, Ohio (1). Convalescent stage sera from these patients were used to aggregate virus particles, thus concentrating them on the microscope grid for easier detection by transmission electron microscopy (89). The IEM technique, developed in the early 1970's, afforded important advantages to the search for viral agents in stools. The ability of convalescent stage sera to aggregate virus allowed IEM to be used both as a serologic assay and for virus detection. The aggregated clusters of virus particles coated with antibody were critical in identifying particles of potential interest and excluding irrelevant particles of similar morphology. IEM also permitted the first means with which to assess the presence of virus-specific antibody in serum specimens, at least on a semiquantitative basis. The sensitivity of IEM was 10-100 times better than normal transmission electron microscopy (22); however, it remained relatively insensitive and its utility is limited (74).

IEM requires highly specialized facilities and technical expertise, is extraordinarily labor and reagent intensive and is unsuitable for the examination of large numbers of specimens (43, 47). Norwalk virus (NV) was identified in stool by IEM in only three of seven outbreaks investigated from 1977 to 1982 in which NV was serologically determined to be the causative agent (68, 69, 70, 92, 101, 104, 157). In two of these outbreaks, NV was identified by IEM in only 1 of 18 and 2 of 30 stool specimens. Reports of better success in detecting NV by use of IEM appear to be the exceptions (69). Because electron microscopes scan a field only 0.000001 meters wide, between 10^5 and 10^6 virus particles per ml of stool must be represent to be detectable (22). Within 48 to 72

hours after onset of symptoms, the virus concentration in stool declines below levels detectable by IEM (159). For these reasons, IEM is impractical for most public health investigations.

Radioimmunoassay and Biotin-Avidin Immunoassay

IEM remained the best method of virus detection for NV until the development of a radioimmunoassay (RIA) technique at the National Institute of Allergy and Infectious Diseases in 1978. The assay relies on the comparison of the binding of antigen to wells coated with pre-challenge serum to binding in wells coated with post-challenge serum of the same subject. Microtiter plates are pre-coated with Norwalk antibody are inoculated with crude stool suspension containing Norwalk antigen. After overnight incubation, they are washed and inoculated with a dilution of the serum to be tested (either pre-or post exposure). After overnight incubation, an indicator consisting of radiolabeled ^{125}I anti-Norwalk IgG (convalescent serum)is added. The plates are incubated and washed, and individual wells are placed in a gamma radiation counter. A 50% or greater reduction in residual radioactivity produced by a serum sample as compared with a buffer control is considered evidence of the presence of NV antibody. Differences in binding between wells coated with post-challenge and pre-challenge serum are measured, and ratios of binding with post-challenge to pre-challenge serum indicate the presence of antigen in the specimen tested (64). Using a modification of this assay, testing stool samples with serum samples of known value, viral antigen was detected in the stools at the onset of illness, but not before, and for a short interval after illness, usually 1-3 days (47, 66).

Antigen detection by RIA is about 10-100 times more sensitive than antigen

detection by IEM (47, 69, 70, 101). In addition to detection of NV antigen, one of the first applications of RIA was for the retrospective detection of antibodies to NV in sera collected during 25 outbreaks that occurred between 1966 and 1977 (67). Ill persons from 11 of these outbreaks had fourfold or greater rises in antibody titer between acute and convalescent phase sera, a recognized standard indicating seroconversion. This study and a subsequent evaluation of further outbreaks of acute infectious non-bacterial gastroenteritis reported to the CDC between 1976 and 1980 suggested that NV was a major cause of such outbreaks in the US (90). The detection of a rise in antibody by RIA has been sufficiently sensitive to provide useful epidemiologic data. In several studies, the number of patients with significant antibody titer rises was apparently reduced by the late collection of acute-stage sera a week or more after the onset of symptoms (8, 105). However, in these studies and others, geometric mean antibody titers in convalescent stage sera were greater in patients that in controls, supporting the conclusion that NV was the cause of the outbreak (68, 94, 104, 111).

Antibody to NV begins to develop within 5 days after the onset of illness. It peaks within 3 weeks and begins to decline by the sixth week post-challenge (74). The early antibody response of IgM and IgA peaks 2 weeks after onset of illness. The presence of pre-existing IgG antibody in about half of the US population precludes the use of single serum specimens to document recent infection in most instances (64). The necessity of obtaining paired acute and convalescent phase sera complicates the use of techniques demonstrating the rise of antibody titer, since it requires repeat contacts with patients after they have recovered from their illness. Patients are often reluctant to provide serum specimens for a mild, self-limited illness from which they recovered weeks before (22).

A disadvantage of RIA is that it utilizes potentially hazardous radioactive isotope-labeled reagents. To eliminate the use of radioisotopes, CDC developed the biotin-avidin immunoassay in 1984, where the ^{125}I label was replaced by a biotin compound which was safer to handle (74). The assay has a sensitivity comparable to RIA. After its initial use of to confirm an outbreak of food-borne NV gastroenteritis in a school in 1984 (76), the biotin-avidin immunoassay became CDC's standard method of testing for NV antigen and antibody for almost a decade (31).

Further shortcomings lie in that the RIA and biotin-avidin assays take six days to perform. Also, antibodies used in the RIA or biotin-avidin assays can detect only the specific strain of virus involved in a particular outbreak, not the full spectrum of NLV agents which have been identified. In a survey of 100 gastroenteritis outbreaks of suspected viral origin submitted to CDC between 1985 and 1988, NV was identified by a fourfold rise in antibody titers in approximately 20% of outbreaks. Approximately 40% showed partial rises (less than half of persons with fourfold rise), suggesting that an antigenically related agent was involved. The remaining 40% showed no titer rises at all, indicating that an agent serologically distinct from NV was involved (22).

Enzyme-Linked Immunosorbent Assays (ELISA)

During the late 1970's and early 1980's researchers continued to develop improved serological tests to detect NLV outbreaks. Like RIA, ELISA was developed for detecting antibody to NV using specimens from experimentally infected human volunteers. Widespread application of these assays was limited by the difficulty in obtaining, purifying, and standardizing human-derived antigen and antibody reagents

(122). In addition, reagents collected from human subjects could be broadly reactive, reflecting the accumulated exposure history of the patients with the various antigenically distinct strains of NLVs (86). For a basic ELISA assay, a 96-well plate is coated with purified antigen and incubated with the human serum to be tested. The plate is washed, removing any unadsorbed human serum. It is then incubated with enzyme-labeled goat anti-human antibody, which adheres to the antigen-antibody complexes. The plate is washed again and the amount of adsorbed labeled antibody is then measured colorimetrically (79, 87).

Recently, a baculovirus vector system was developed which could synthesize NV capsid protein (83, 87). The recombinant-expressed Norwalk virus (rNV) capsid protein self-assembles into empty virus-like particles. This method represented a major breakthrough because large quantities of specific, standardized, purified capsid antigen reagent could be generated with relative ease for large-scale laboratory use without passage through human subjects. These particles are useful as antigens to produce hyperimmune serum for use in a highly sensitive ELISA for detecting NV antibodies in experimental volunteers and in epidemiologic investigation (57, 62, 87). Similar ELISAs have since been developed for the Snow Mountain and Hawaii viruses, representing two other antigenically distinct genogroups of NLV (113, 122).

Because these tests were originally designed to use human convalescent phase serum from patients, they were of relatively low sensitivity because of the low affinity of the antibodies for the viruses (83). In addition, sources of serum were limited. The use of rNV has permitted the generation of hyperimmune serum to NV in animals (87). The hyperimmune serum and baculo-virus-produced rNV were in turn used to develop highly

sensitive ELISA tests for detection of NV-specific antigen and serum antibody. Graham et al (57) reported that using rNV generated reagents, the sensitivity of ELISA for detecting rNV in serial dilutions of purified antigen was comparable to RT-PCR. The ELISA is also highly specific; the NV antigen ELISA failed to detect Snow Mountain agent, Hawaii agent, HuCV, Sapporo, and astrovirus, as well as feline calicivirus, poliovirus and other enteric viruses (57, 62). Thus the high sensitivity of the new ELISA techniques and the unlimited supply of hyperimmune sera and NV capsid antigen made possible by the use of rNV permitted research to move forward. Shortly after, the capsids of Mexico virus, Southampton virus and recently Lordsdale virus have been cloned using the baculovirus system, enabling a wider range of strain detection.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The use of molecular epidemiology methods to characterize NLVs was made possible when Jiang et al (83) sequenced the entire genome of NV in 1992. Using Norwalk virus-specific complimentary DNA (cDNA), Jiang et al (83) showed that NV contains a polyadenylated single-stranded RNA genome of about 7.7 kilobases. Based on this knowledge, they developed a reverse transcription polymerase chain reaction (RT-PCR) method for the detection of NV in human stool, a method in which enzymes are used to amplify the presence of viral nucleic acids. Other researchers have subsequently developed improvements in technique, and identified new primer sets based on conserved gene sequences in other NLV genogroups, including GII Southampton virus and Lordsdale virus, which are useful targets for amplification (4, 121).

Polymerase chain reaction assays are conducted as follows. Briefly, the stool specimen is filtered, denatured, and lysed to purify and isolate the viral RNA, which binds to a silica-based membrane. Individual primers or sets of primers targeted at specific conserved regions in the capsid and RNA polymerase gene sequences of the viral genome are selected, and added to the reaction tube with enzymes. Precisely timed cycles of heating and cooling cause replication of the gene sequences and the number of amplicons increases logarithmically. Gel electrophoresis separates the amplicons into bands based on their identifiable, specific number of base pairs. The formation of the signature bands indicates presence of virus (119). High quality purification of the viral RNA is very important for obtaining positive results (83). It has been found that small particles and soluble substances intrinsic to the material being tested may inhibit the PCR reaction (83, 133).

The sensitivity and specificity of this method is exquisite. Jiang et al (83) estimated that stool samples may contain approximately 10^5 viral particles per ml, and could detect NV RNA in stool samples diluted up to 10^{-4}, this suggests a detection limit of as few as 10 particles and certainly 100-1000 particles. Because specific conserved regions represent the "fingerprint" of the virus, using only one set of primers would detect only one target strain. Ando et al (4) simultaneously used sets of primers which would differentially detect several strains belonging to the antigenically distinct Genogroups I and II. This allowed a wider range of virus identification and formed the basis of techniques now practiced in diagnostic laboratories throughout the US (Steve Michalik, MDCH, personal communication).

Testing Food Specimens

In 1993, Atmar et al (6) first adapted RT-PCR techniques to attempt to recover viruses from experimentally contaminated oysters. In separate assays, they were able to detect as few as 9-90 virus particles in serial dilution of stock virus lysate, 300-3000 virus particles after the addition of virus to oyster extract, and about 4100-41000 virus particles in bioaccumulating oysters. When oysters were seeded with NV extracted from a human stool specimen, they estimated they were able to detect 50-500 virus particles in the oyster homogenate. More recently, a multiplex RT-PCR was developed for the simultaneous detection of human enteroviruses, represented by poliovirus, Hepatitis A, and NV. Three different sets of primers were used to produce three size-specific amplicons for the respective viruses (6). When tested on serial diltuions of mixed, purified virus suspensions, the method achieved detection limits of 1 PFU for HAV and poliovirus, and 1 RT-PCR-amplifiable unit of NV. When perfected, this method might enable rapid and cost-effective assays of foods by allowing testing for different strains at the same time. It is of note that tests were not run on food samples, but in simple suspensions.

There are serious limitations in the present use of molecular biological techniques that can reduce their usefulness in measuring the virological safety of foods. Compared to bacterial pathogens which can actively grow in foods, enteric viruses are merely harbored by them. These viruses are present in foods only as a result of incidental contamination during production, processing, preparation or service. This presents a sharp contrast to clinical specimens where viruses occur in vast numbers, being concentrated within the human body through active replication. In foods, viruses may be present in minute quantities; numbers sufficient to cause illness but below the detection limit of the RT-

PCR, due to factors intrinsic to the food specimen which interfere with detection. Current PCR methods cannot yet consistently overcome these intrinsic factors, and are not yet capable of reliably detecting these small but infective numbers of viruses.

The viability of the virus is an important issue in food quality control. The virus may be inactivated due to partial degradation during storage, processing, or by other means (144). While NLVs have been shown to be resistant to inactivation by heating at 60°C for 30 minutes, resistant to chlorine, exposure to salt water, the UV light used in oyster depuration, and extended periods of freezing, it is uncertain the degree to which the viruses that are inactivated by damage to the capsid protein maintain their intact genomic structure (45, 47, 98, 99, 116, 119). Viruses sustaining damage to their capsids may test positive by PCR even though they are not infectious (144). Likewise, the nucleic acids may also be damaged by environmental stressors. This has different implications for routine quality control testing as compared to outbreak investigation. For food quality control testing, the presence of inactivated virus would represent false-positive detection, possibly resulting in the condemnation of virologically safe foods.

By contrast, virus-containing extracts from foods may contain extraneous material, such as acidic polysaccharides, glycogen, and lipids that inhibit RT- PCR reactions. Such is the case when enteric viruses are extracted from shellfish. Further, techniques used on shellfish have not been tested on shellfish from different geographical areas and different seasons when the levels of compounds such as glycogen, lipids and algae are especially high. The presence of these inhibitors affects the sensitivity of the test and has been shown to cause false-negative results (6, 144).

In summary, laboratory techniques for detecting viruses in foods are still developing. Concentration methods are usually cumbersome, and yields are less than optimal. Immunofluorescent staining is expensive, slow, and requires highly trained personnel. Alternative methods such as ELISA and RT-DNA probes have been tested but are limited by high detection limits ($>10^3$ infectious units), unavailability of reagents, and poor sample quality. Despite enormous strides in the ability to detect human enteric viruses in foods using PCR, the technique is still limited by the absence of effective concentration methods, the presence of enzymatic inhibitors, and the ability to distinguish between infectious and noninfectious virus particles (82).

RT-PCR and Phylogenetic Analysis

NLVs possess single stranded RNA genomes and were finally classified definitively as members of the family Caliciviridae through sequencing of viral genomes and RT-PCR analysis (37-40, 62, 83). The availability of the complete cDNA nucleotide sequences of two NLV strains, Norwalk virus (NV) and Southampton Virus (SOV) has allowed the development of primers for the detection of NLVs in fecal samples by PCR. Detection of different strains NLV have been reported using NV-specific primers and "degenerate" primers to accommodate sequence variation between different NLV strains (3, 4, 62, 63, 128). On the basis of sequence variation within the RNA polymerase gene, the capsid gene and the 3' end open reading frame (ORF2) (3, 4, 107), it has been shown that NLVs can be divided into at least two major genetic groups, based on gene sequences in the RNA polymerase region of the genome. Genogroup I (GI) includes two subgroupings: the Norwalk Virus cluster, containing the viruses named Norwalk (NV), Southampton

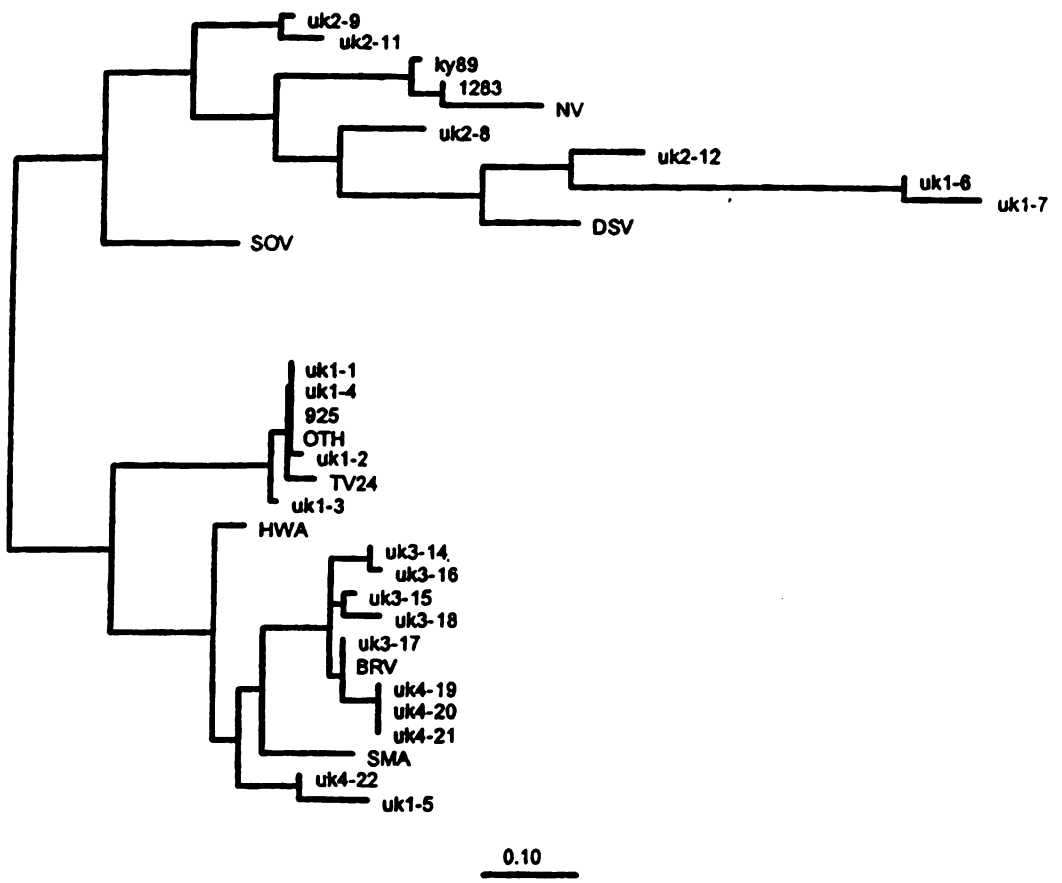

Figure 1 - Dendogram of phylogenetic relationship between HWA, Hawaii Agent and 30 other strains of NLV. The length of the abcissa to the connecting node is proportional to the genetic distance between sequences. DSV, Desert Shield Virus; SOV, Southampton Virus; BRV, Bristol Virus; NV, Norwalk Virus; SMA, Snow Mountain Agent. Line indicates 10% variation in nucleotide identity. From Ando et al (4).

(SOV), Cruise Ship Virus (CSV), Desert Shield (DSV), Venlo (VV), and many other numbered strains such as UK2; and the Gwynedd Virus (GV) cluster, containing UK-1 and other numbered strains. (Note that subsequently, Noel et al (128) placed GV in to Genogroup II based on sequence data in the capsid region. Genogroup II (GII) contains 5 subgroups; the Toronto Virus (TV) cluster, the most closely related to the Genogroup I, including Mexico virus (MV); Hawaii Virus group (HV), sharing only about 48% amino acid identity with NV; Lordsdale Virus cluster (LV), a large group including LV, Bristol (BRV) and Camberwell (CAV) viruses; and the Snow Mountain Agent (SMA) cluster, containing SMA, Melksham (MK) other numbered viruses (3, 4, 50, 107, 128). Figure 1 portrays a dendogram showing relatedness of various strains of NLV by pairwise nucleotide sequence identity (4). By characterizing and analyzing the amino acid and nucleotide sequences of the various distinct stains of NLVs it becomes possible to study their distribution and occurrence in outbreaks and sporadic cases, and to elucidate the epidemiology of NLVs.

Several studies demonstrated that strains belonging to GI and GII are not only genetically distinct but are also antigenically distinct. (107). Noel et al (128) observed very little cross-reaction in patient seroresponses to the expressed antigens between the two genogroups. However, the seroresponse to the expressed capsid antigens in subjects infected with GI strains differed from the seroresponse in those infected with GII strains. Patients infected with NV, SOV, CSV and DSV, strains which showed up to 37% nucleotide and 38% amino acid divergence from prototype NV, demonstrated relatively homogenous seroresponse to the single NV antigen. This contrasted with seroresponses in those infected with GII strains. For example, patients infected with TV and HV strains

exhibited good seroresponses only when the infecting strain showed less than 22% nucleotide and 6.5% amino acid divergence from the respective antigens. Crossreactivity has also been shown between HV and SMA (113). Studies in Brazil have shown that infection with and immune response to one strain often does not confer protection against infection with a different strain within the same genogroup (137).

Ando et al (49, 100) used first RT-PCR techniques to investigate an large multistate outbreak of NLV illness associated with oysters from Grand Pass/Cabbage Reef, Louisiana. Using RT-PCR and other techniques, NLV strains of the P1A phlyogenetic group (GI) were detected in 32 (86%) of the specimens, from 8 clusters in 4 states. Of the 32 PCR products obtained, 19 (59%) from all 8 clusters had an identical genetic sequence. Given that these 19 identical products were detected from geographically separate areas, but epidemiologically linked to the same contaminated oysters, they concluded that the 32 products detected could only be represented by a single strain from a common source which was responsible for this large outbreak (3).

Sugeida (155) successfully used nested-RT-PCR to detect two different genotypes of NLV from stool and from oysters implicated in an outbreak of NLV foodborne illness in Shizuoka Prefecture in Japan. This finding confirmed the epidemiological link of the illness outbreaks with the oysters, and the nested technique permitted detection of two distinct genotypes of NLV coexisting in the same specimen.

When a large number of outbreak strains were characterized using RT-PCR and genetic sequencing by workers at CDC, it was found that strains from 2 outbreaks which occurred in the same area in Arizona 53 days apart, and strains from 2 outbreaks that occurred 74 days apart in South Carolina, differed by 2 and 8 nucleotides respectively, and

by 0 amino acids. This suggests that the same stain may persist in the same community and result in repeated outbreaks. It also suggests that the genetic sequence may drift with continued passage. Over time, they may accumulate more and more sequence changes, first as silent changes in the third base position, then as coding differences as well (127). Thus NLVs possess a mechanism to cause the phylogenetic tree of NLVs to continue to grow and change, with different strains emerging and becoming predominant. The speed at which such genetic drift may occur is currently under study. It has been shown in one instance that drift did not occur after 4 serial passages through volunteers (C. Moe, personal communication).

Molecular analysis of NLVs associated with outbreaks has demonstrated both a great diversity of strains in circulation and the presence of a single predominant strain during given time periods (50, 106, 128, 165, 166). Partial genetic sequences now exist for over 100 strains of NLV. Despite this, few surveillance programs use PCR techniques for the detection of NLV's and fewer still characterize the NLV strains that could potentially link outbreaks to a common origin (127). Only about 17 State and regional laboratories routinely use RT-PCR to detect NLVs at this time.

NLVs were first found naturally occurring in animals other than humans by Sugeida in 1998 (155), then subsequently in cattle by others. In a paper published in 2000, van der Poel et al (164) reported that using RT-PCR techniques and genome sequencing, they found that three phylogenetically different strains of calf caliciviruses formed a tight cluster closely related to human NLVs from Genogroup I. The nucleotide sequence identities between the calf NLVs and GI NLVs were 63-70%, with 75-77% nucleotide homology. Swine NLVs were clustered as a separate lineage within

Genogroup II, with 69-71% nucleotide and 79-83% amino acid identity. The calf and pig strains did not hybridize with the probe mixture used to detect human NLVs. However, the genetic distances between the animal and human NLVs are similar to the distances between the GI and GII human strains (164).

The close similarity of porcine and bovine sequences to the NLVs infecting humans indicates the possibility of an animal reservoir for human infection. This raises two important issues. The first is that epidemic spread of NLVs from an animal reservoir may be possible. Secondly, these findings may at last lead to the development of an animal model for the NLVs of humans; a pig model would enable studies of mucosal immunity following NLV infection (164).

Chapter 4

SEROPREVALENCE

Once the Norwalk agent was identified in the 1970's, several researchers set out to characterize the prevalence of the NV in various populations. Detection of the virus was dependent upon the sensitivity and specificity of the means of identification; radioimmunoassay (RIA) examination of serological specimens was the best method available. At this time, the detection was targeted at prototype Norwalk virus only; the genotypic and antigenic differences of other NLVs circulating had not yet been characterized.

Greenberg et al (64) conducted the first study of NV seroprevalence in various countries using RIA techniques. They tested serum specimens from urban volunteer blood donors in the US, Belgium, Switzerland and Yugoslavia; specimens from a longitudinal study of enteric virus infections from villages in Bangladesh; specimens from Ecuadorian Indians, including the highly isolated Gabaro tribe; healthy Nepalese villagers; healthy Yugoslavian children, from specimens collected for a serological survey for HAV; US children's samples, from hospital admissions for respiratory and other non-gastrointestinal complaints; and from US adult male and female homosexuals, as part of another serological study.

Antibody to NV was found in serum from individuals in all of the areas studied. Of the 861 persons tested, 71% had antibody. There was not a great difference in antibody prevalence in male and female adults, nor between Western countries (US, Belgium, Switzerland,) and the less developed countries such as Nepal, Bangladesh, and

Ecuador. The prevalence of NV antibody among homosexuals did not differ significantly from the other US blood donors (64). It was found that by the fourth decade of life, antibody acquisition was at least 65% in all countries studied.

Of interest was the NV antibody prevalence obtained in the Gabaro tribe of Ecuador. This tribe lived in an extremely isolated village and had little if any contact with neighboring tribes or Westerners prior to 1972, and very infrequent contact since then. The inhabitants of the Gabaro village have had some hostile contact with other Indians and outsiders, but this has not been of a sustained nature. None of the 16 adults or children had an antibody response to NV indicating prior infection. The Gabaro are felt to be closely related to the surrounding Indian tribes, so their lack of antibody was thought unlikely to be due to a genetic influence. Apparently, NV had not circulated in this isolated group in the 40 years prior to testing. Perhaps a small population cannot sustain the virus. Greenberg et al 64) proposed that the virgin antibody status of this population is evidence against a common animal vector for NV, although it is was not stated which animals are "common" in remote Amazon Indian villages. A later study on isolated Amazonian Indian tribes in Brazil using a more sensitive enzyme immunoassay showed that seroprevalence ranged from 39% overall in one tribe to 100% in several others, two of which first contacted Westerners in 1970 (52). Reasons for this could be that host differences actually exist, that the earlier methods were not sensitive enough to detect the antibodies, or that other strains of NLV might have been present instead.

Poor sanitation conditions are likely contribute to earlier and more prevalent infections, which may account for differences in the rates between developed and developing countries. However, these studies show that NV infection is a common and

Table 2. Age distribution of healthy adults tested worldwide and percent testing positive for anti-NV IgG antibodies.

Country	Age (years) 18-19	20-29	30-39	40-49	>50
South Africa Taylor (158) n=579	47.2%	65.9%	62.1%	62.1%	-
Singapore Numata (129) n=50	63	65	-	64	-
England Gray (59) n=1976	74.9	81.4	85.5	91.6	87.9
Japan Numata (129) n=380	76	80	89	98	-
Sweden Hinkula (77) n=122	88.2	80	87.5	89.4	94.7
Indonesia Numata (129) n=90	90	100	-	92	-
Australian Aborigine Parker (136) n=38	100	100	90.9	100	90.9
Papua New Guinea Numata (129) n=50	100	100	100	100	-
Kuwait Dimitrov (46) n=222	100	100	100	100	100

Note- to permit more accurate comparison, all studies cited above used ELISA methods.

Note- serum specimens cited here were tested only against prototype Norwalk Virus capsid antigen.

recurring condition throughout the world. The prevalence rate of ≥70% found in most adult populations might reflect a steady-state condition in which reinfection with an antibody response balances the disappearance of the antibody (64). Table 2 portrays the seroprevalence of Norwalk virus infection among adults in several countries, as detected using ELISA. Figure 2, a chart titled "Seroprevalence of NLVs" , highlights the differences in prevalence of two strains of NLV in two developing countries, Indonesia and Papua New Guinea, and Singapore, which enjoys a much greater level of economic development and sanitary infrastructure (86, 136).

As the genomes of other NLVs were sequenced in the late 1990's, it became possible to produce recombinant capsid antigen for other strains of NLV. Seroprevalence studies were conducted using recombinant capsid antigens from Mexico virus (rMX), Hawaiivirus (rHV), and Lordsdale virus (rLV), all belonging to GII ; and Southampton virus (rSV) and rNV (GI strains), to see what relationships might exist. When seroassays for NV and MxV were conducted on samples from 222 Kuwaiti adults, the prevalence was 100% for both viruses in adults aged >20 (46). In Singapore, while the rate of NV was very moderate at 63% among subjects aged 20-29 and 65% in those aged 30-39 yrs, the rate of MxV antibody detection was 73% and 95% in the respective age groups (79, 129). Among Canadian subjects tested, HV antibody was predominant in subjects <20 years of age, when the prevalence of NV seropositivity became predominant (38, 39). In Japan, MxV was more prevalent than NV in all age groups <50 years of age (79). In Italy, LV antibodies were present in >93% of subjects of >5 years of age, while SV was present in 26-38% subjects in age groups ranging from 1 year to>70 years (140). Studies of subjects from London, England, and in UK showed that MxV infections occurred

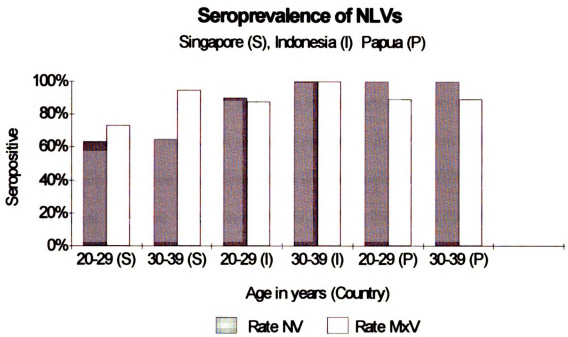

Figure 2 - Seroprevalence of NLVs in Singapore, Indonesia and Papua New Guinea

earlier in life, affecting children during the preschool years, whereas NV was seen to occur later, among older children and adults (39, 136).

The predominance of virus strains may vary widely in different regions within the same country (50,126, 127, 131,137). In Chile, a study was conducted comparing the rates of seropositivity for MxV and NV in Punta Arenas and Santiago, cities separated by hundreds of miles. The overall seropositivity for NV in Santiago was 83%, whereas in geographically distant Punta Arenas 65% were seropositive for NV, a statistically significant difference. The rates of NV vs. MxV seropositivity also differed significantly between the two cities: 91% in Santiago compared to 76% in Punta Arenas ($p<.001$, χ^2, Yates corrected). Note that study populations included similar proportions of subjects from low, middle and high socioeconomic groups (131). Cubitt and Jiang (38) report that strains of MxV and UK3 NLVs predominated in different areas of the UK during the same year. In a study of Amazonian Indians of Ecuador, 4 isolated tribes had a NV seroprevalence of 100% among adults, while 4 other isolated tribes had NV seroprevalences of 50%, 55%, 30% and 67% (52). Figure 2 depicts the differences in seroprevalence between two strains of NLV in three Asian countries. The varying levels of exposure may be attributed to differences n the predominant strain of NLV circulating in that general time period. It appears that Singapore's greater level of economic and infrastructure development my be reflected in seroprevalence rates which are similar to those in more developed Western countries.

The major differences in prevalence between countries lie in the age at which infections and antibody are acquired. In the pediatric populations studied, children from the developed world including the US, Japan, UK, and South African whites generally

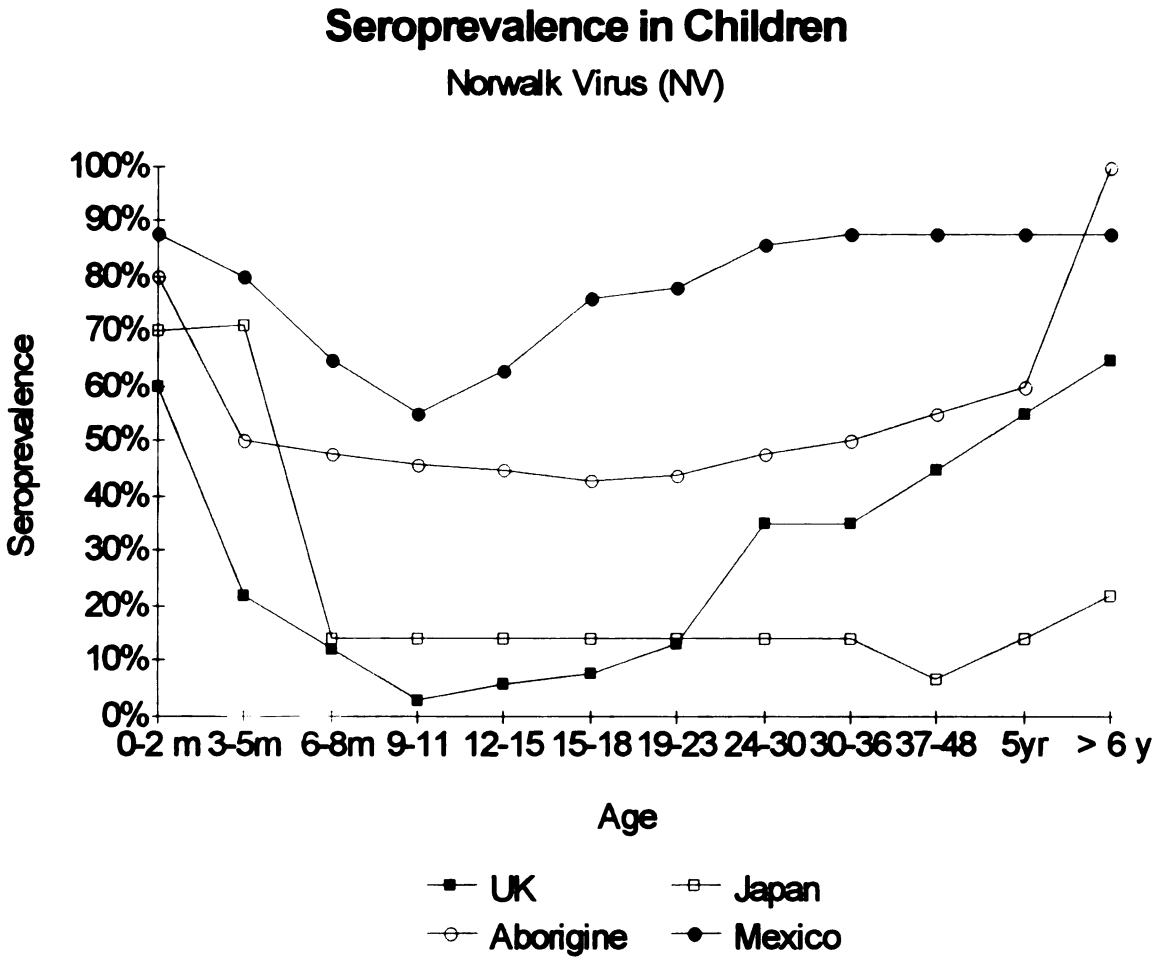

Figure 3 - Norwalk Virus Seroprevalence in Children

acquired antibody more slowly than did children from developing different age groups in developing countries like Ecuador, Bangladesh, Mexico, and among South African blacks (11, 59, 64, 86, 136, 157). Children are born with IgG antibody roughly equal to that of the mother. The maternal antibody disappears by the fourth month of life (136). Antibody levels then rise after the initial infection with NLV. Studies show that NV infection appears to be relatively uncommon in early childhood in developed countries like the United States and Japan, while in many developing countries, primary NV infection occurs very early in life (12, 65). In Mexico and among the Cuna Indians of Panama, less than half of the study children of less than 2 year of age had antibody to NV, but by age five, 98% had antibody (86, 142). In these cases, there is no obvious window between the decay of maternal antibody and primary infection, depicted in Figure 3 as a lack of a steep trough after maternal antibody is gone but before the primary infection occurs (157). What proportion of these differences is due to geographical location, population densities, sanitary facilities, cultural practices or other factors has not been clearly established.

Prevalence studies among children in the developing world confirm that primary infection tends to occur very early in life (47, 64). Figure 3 illustrates the dramatic decline of maternal antibody at about 6 months of age. In children from UK and Japan, representing developed countries, the rate of seropositivity remains low for several months, indicating that the rate of primary infection is lower. The less evident decline of maternal antibody among Mexican and Aboriginal children indicates a high rate of primary infection with NLV at a very young age. By the age of 6 years, the level of seroprevalence to NLV in Mexican and Aboriginal (and interestingly, British) children equals the level in adults. By contrast, seroprevalence in Japanese children does not reach

adult levels until the late teenage years (79, 129). Children in the US and Japan acquired primary infection at a much slower rate, approaching adult levels much later, during adolescence (59, 136). Paradoxically, a study of healthy children in Finland found that 73% were positive for NV IgG antibodies by the age of 23 months, compared to a study in Sweden where seroprevalence was 25% in children of $\leq$ 5 years of age (77,109).

The use of sera collected for a longitudinal study of infectious diseases and nutrition in rural Bangladesh allowed Black (10) to conduct seroprevalence studies for NV and also to describe the annual incidence of NV there. The prevalence of antibody to NV in children 3-6 months was 7% (after the decline of maternal antibody), and increased rapidly to 100% in four year-old children. The incidence of seroconversion ($\geq$4-fold titer increases) was highest in 1-2 year-olds and in children who had low or undetectable levels of antibody. The annual incidence of NV seroconversion was 29% among children of less than 50 months of age. Overall, 46% of the children developed NV infection during one year. The infection was most common during the December-March period, representing the cool, dry period in Bangladesh, although moderate rates of transmission were observed year-round (10, 41).

Distinct strains of NLV have appeared to predominate by age group. Comparison of the acquisition of antibody to rMxV and rNV antigens indicates that in UK, MxV infections occur earlier in life affecting children during the preschool years, while NV occurs later, predominantly among adolescents and adults (38, 135).

Seasonal prevalence was the first epidemiological descriptor for NLV infection: it was christened "winter vomiting disease" by Zahorsky (1), who first described the illness in 1927. Longitudinal data show that outbreaks of NLV illness are most common from

October through May, and one strain of NLV usually predominates among strains co-circulating in a geographic area (106, 110, 127). It has been observed that the strain which is predominant during the high season was often completely absent during the preceding high season. Lewis (110) reported that the detection of 4 isolates of MxV from British children in the summer of 1993 heralded the major epidemic which occurred in the fall of that year. A similar pattern was reported in the Netherlands during the same time period (165, 166). Volunteer studies have shown that immunity to NLVs is short-lived; thus herd immunity following an epidemic may play a role in the dynamics of NLV prevalence (110). It appears that over the winter season, infections with the predominant strain are common and only a subset of patients actually requires medical attention which leads to establishing a definitive viral diagnosis. During this period immunity to the specific genotype may develop throughout the population and the prevalence of infection with that genotype then declines and is superceded by a different genotype (106).

Recently, Noel et al (127) described the global identification of a common strain of NLV in the Lordsdale virus (LV) cluster. Prior to 1995, the nucleotide sequence in the RNA polymerase region in NLV outbreak strains was so diverse that no two strains from different outbreaks in the US were identical. In 1995, this changed when strains from 60 out of 109 (55%) dispersed and apparently unrelated outbreaks were founds to be very closely related; 39 of the 60 (65%) had identical sequences, and an additional 21 differed by only 1-4 nucleotides in this region. Further inquiry revealed that the same strain had been identified in UK in February 1995, in Brazil, the Netherands, Canada, and Australia in late 1995; Australia, the Netherlands and China in 1996, and in Germany before October 1997. It is not understood how this strain spread to geographically distant

locations in the US and abroad with no obvious common source of exposure and what modes of transmission or selective factors allowed the sudden emergence and rapid global spread of this strain (127, 150, 156, 165, 166).

Seroprevalence information must be interpreted with an eye to several poorly described factors. Blacklow et al (11) have suggested that either a genetic factor is involved in protective immunity or repeated exposures to the virus are necessary to elicit a protective immune response. Future research is needed to clarify the nature of both of these important aspects. More research is needed to clarify the amount of crossreactivity possible between specific NLV antibodies and other phlyogenetically closely related NLV. Varying levels of crossreactivity between NV and other NLVs from genogroups have been shown in adult volunteer studies (160). Also, human antibodies vary in their affinity to bind, and antibody response to infection varies widely between individual subjects (83).

Nonetheless, it appears that NLV infection is ubiquitous, and although antibodies are acquired early in life, the role of NLVs as pediatric pathogens has yet to be elucidated. More research is needed to describe the mechanisms responsible for resistance or susceptibility to NLV.

Chapter 5

MODES OF TRANSMISSION

Foodborne contamination with NLVs is an important mode of transmission. Foods can become contaminated either at source (primary contamination) or at the time and place of preparation (secondary contamination)(5). Molluscan shellfish are thus far the only foods which have been found to be contaminated with NLV at the source. Other potential sources of primary viral contamination are the application of sewage-polluted ground or surface water during irrigation and fertilization. The growing trend toward the land application of municipal sewage sludge could also be a source of contamination to fruit and vegetable crops. It has also been suggested from experimental studies that mammalian viruses might be taken up through the roots of plants (5). Viruses could conceivably be harbored in stem scar and bud tissues. They also might conceivably be drawn through the skin into fruits and vegetables along with rinse water when a temperature gradient between the water and the fruit exists, as has been shown to happen with coliform bacteria in tomatoes and other fruits. However, no outbreaks of NLV illness have yet been traced to these sources. NLVs might also be borne to hand-harvest food crops by the hands of farm laborers. While this source of contamination has been demonstrated for bacterial and parasitic pathogens such as *E. coli O157:H7* and *Cyclospora cayatenensis*, the association has not yet been reported for NLV. In most cases when food items such as lettuce were implicated in outbreaks of NLV illness, infected food handlers were also implicated.

Other important modes of transmission are sewage-contaminated water and person-to-person, including aerosols traveling through the air to contaminate surfaces,

which will be discussed in detail.

Contaminated Shellfish

A significant source of NLV illness is bivalve mollusks, including oysters, clams, cockles, and mussels. Because they are filter feeders, they are able to concentrate any viruses which may be present in waters of the shellfish beds due to sewage contamination. In Britain, 169 outbreaks of illness associated with mollusks contaminated at the source occurred between 1965-88; of these, 138 were confirmed or consistent with NLV viral gastroenteritis (5).

The consumption of fecally contaminated shellfish has long been associated with outbreaks of illness caused by enteropathogens, including NLVs. Raw shellfish have been implicated in outbreaks of foodborne viral gastroenteritis in several countries, including the U.S., Europe, Australia and Japan (35, 55, 69, 70, 155). Most of the commonly eaten shellfish, including oysters (23-26, 35, 49, 69, 70, 155) , clams (124), and cockles (49) have been vehicles of transmission for viral gastroenteritis. Norwalk-like viruses have been the most common agents identified in outbreaks of oyster-associated gastroenteritis.

A recent review of seafood-associated disease outbreaks in New York for the period 1980-1994 offers a perspective on the proportion of illnesses caused by NLV in seafood (167). Outbreaks in which a Norwalk or Norwalk-like viral agent was suspected based on epidemiologic profile, but not confirmed, were classified as caused by a "gastrointestinal virus". During this time period , the etiologic agent was confirmed for 654 (36%) of 1802 foodborne outbreaks, and 148 (44%) of the 339 seafood-associated outbreaks. Of the seafood-associated outbreaks, 14 (9%) were attributed to bacteria, 69

(47%) to viruses, and 65 (44%) to chemical agents, such as ciguatoxin and scombrotixin. Seafood vehicles accounted for 85% of foodborne outbreaks caused by Norwalk virus. In the 192 outbreaks where an agent was not confirmed, 129 (67%) suspected agents were compatible with NLV. Shellfish accounted for 64% of the seafood-related outbreaks, and all 204 outbreaks caused by confirmed or suspected viruses were associated with shellfish. Norwalk or NLVs were confirmed or suspected in 94% of those shellfish outbreaks. Of the 216 shellfish-associated outbreaks, 210 (97%) were attributed to raw or lightly cooked foods (167).

A series of major outbreaks associated with the consumption of raw oysters occurring in the 19th and early 20th centuries, including one in 1855 that led to the deaths of several "highly esteemed" citizens of New York from cholera, an outbreak of typhoid fever at Wesleyan College in Connecticut in 1894, and a typhoid epidemic in 1924-25, led to the development of the National Shellfish Sanitation Program (NSSP). This cooperative program, involving FDA, State regulatory agencies and the shellfish industry, is charged with controlling the safety and quality of shellfish shipped in interstate commerce. Recommendations, such as limiting harvests to areas with clean water (<14 fecal coliforms/100ml water), depurating harvested shellfish to reduce bacterial counts below the market guidelines (230 coliforms/100 g meat) and requiring tags naming the location and date of harvest on all boxes of shellfish sold to allow back-tracing of contaminated lots and identification of the contaminated shellfish beds, have targeted the continuing problem of shellfish safety (49). NSSP standards require fecal coliform testing of shellbed waters from various points at least once a month (25).

Oysters are filter feeders, and are believed to concentrate virus in the midgut gland (155). It has been suggested that a period of depuration, wherein oysters are held in a tank of disinfected sea water for a time to reduce the concentrations of harmful organisms within the gut, might reduce overall levels of contamination (35). Experimental findings suggest that oysters may be able to rid themselves of 99% of enteric viruses in 25 days under laboratory depuration conditions (100). Unfortunately, this time period for depuration is not economically feasible for the shellfish industry. In Australia, the state of New South Wales since 1979 has mandated a 36 hour period of depuration; the Australian government is now evaluating this for national legislation. England and Wales have adopted the EU Shellfish Directive 79/923/EEC, which mandates depuration of oysters grown in any but the most microbiologically pristine (Class A) waters.

Regardless, outbreaks of NLV illness have been associated with depurated oysters (35, 55 69). Gill et al (55) describe a large outbreak of gastroenteritis associated with consumption of raw oysters which had been depurated for 72 hours in seawater. The depuration tank held 5500 liters of seawater and about 8000 oysters. Water was circulated continuously through a 30 watt ultraviolet light sterilization plant at 2730 liters per hour; the average depuration rate was 0.35 liter/oyster/hour. The tank water was recirculated through the ultraviolet light for 48 hours before the introduction of the oysters. Depurated oysters had been independently tested by a hospital laboratory on behalf of local authorities for 5 years prior to the outbreak; of the 78 samples tested at regular intervals, only three had more than 5 fecal coliforms per ml and two of the three samples had >15 fecal coliforms per ml. Studies of marine caliciviruses, more closely related to human Sapporo virus than to NLVs, have shown that shellfish exposed to

marine caliciviruses and held at less than 10° C in a continuous flow of sterile sea water still retained virus 60 days later, when samples were seen to grow in mammalian cell culture. The marine caliciviruses remain viable for more that 14 days in 15° C sea water (152). While this model may not fully represent the fate of NLVs in sea water, nonetheless it appears that they can survive and persist in shellfish beds for relatively long periods of time.

The use of coliform counts as an indicator of quality of water in the harvesting area has often failed to predict contamination of oysters with NLVs (25). In a large outbreak involving several states stemming from Louisiana oysters, the oysters of acceptable bacteriological quality were harvested from waters meeting NSSP standards of quality (49). In an outbreak involving oysters from Appalachicola Bay, Florida, the oysters were taken from areas with acceptable water quality; water was sampled from 39 monitoring sites in the bay three times in a two month period. No environmental source of pollution was identified. Sanitation procedures at the oyster processing facilities where seafood dealers purchased oysters also met the applicable sanitary standards (26).

Another incident, again involving Louisiana oysters, occurred in 1996-7. Investigators interviewed 15 of 20 implicated oyster harvesters, and inspected 8 of their boats. Seven of those boats had inadequate sewage collection and disposal systems, and harvesters admitted to routinely discharging their sewage overboard. In a previous outbreak it was found that harvesters ill with NLV gastroenteritis had disposed of sewage overboard and contaminated a broad area of oyster bed (100). Sewage from off-shore oil rigs has also been the source of sewage contamination of shellfish beds (27).

In the 1996-7 outbreak, the implicated shellfish beds lay 12-15 miles from the

nearest community sewage outlet, recreational boating was infrequent in December, commercial boating traffic was infrequent because of the shallow depth of the water, and all oil rigs were considered to have adequate sewage facilities. Molecular analysis of stool samples from the six outbreak clusters identified three different strains of NLV which were associated with three geographically separate harvest sites. Researchers suggested that different harvesters who were infected during the same time period with the genetically distinct strains of NLV, and each of whom dumped their sewage in different waterways, possibly during favorable environmental conditions (low temperature and lowered salinity due to an influx of diverted fresh water), caused contamination of oysters with NLV (27).

In an earlier Louisiana outbreak associated with oysters from Cabbage Reef, Kohn et al (100) calculated that, if we assume an infectious dose of 10 virus particles and if we assume that a 1 ml sample of stool from an ill person at 10^5 dilution is still infectious, then stool from an ill person contains at least 10^9 virus per liter. If we further assume that an ill person's output of stool is 1 L per day, that an oyster contains 25 ml of water, and that an oyster can concentrate enteric viruses 50-fold (conservative estimate), then stool from a single infected person over 1 day would be enough to contaminate an oyster bed containing 2×10^8 liters of water. This is equivalent to a reef area 1 km long by 100 m wide and 2mm deep, approximately half of the size of the entire Cabbage Reef harvest area (100).

Rainfall, reduced salinity, total and fecal coliform counts, pH, turbidity and sediment total /fecal coliform counts are all associated with the presence of viruses in water. Other significant factors such as currents, and geophysical factors such as bottom

topography, depth, inflow changes, and shoreline contours affect the rate and pattern of the flow of water from the source of contamination, making prediction of dispersal difficult. This complicates our understanding of the true relationship between virus survival and the effect of these environmental factors on the rate and range of distribution of the virus in the environment (54, 118).

A recent perspective by Smith et al (152) describes the emergence of caliciviruses from ocean reservoirs causing mammalian disease. This is exemplified by the emergence of highly contagious vesicular exanthema of swine, caused by the feeding of infected raw fish offal to swine, which caused devastation of the California swine industry in the 1930's. In another instance, shellfish beds on US coasts were positive for caliciviruses of unknown type when tested with a cDNA calicivirus group-specific hybridization probe from a marine calicivirus (San Martin Sea Lion virus SMSV-5), a virus which can infect fish of commercial value and has infected humans, causing blister-like lesions. This review raises interesting questions on the role the ocean might play as a reservoir to other caliciviruses which have been known to cause zoonoses (152). Might the ocean also retain NLVs for indefinite, protracted time periods, or indeed as act as a reservoir?

While most outbreaks are attributed to oysters eaten raw ("oysters naturelle") (35, 49, 155), the survival of NLVs in lightly cooked shellfish has also been documented. Several incidents have resulted from oysters served steamed (23, 25, 26, 49), and roasted (26) and from steamed clams (124). In a study by Dowell et al (49), the attack rate for oyster eaters at a large festival was 54% for those who ate steamed oysters only, while it was 56% for those who ate both raw and steamed oysters. In the widespread shellfish-related outbreaks in New York, attack rates of 26% occurred among those who ate only

steamed clams, and 56% among those eating both raw and cooked clams (124). Similarly, at two Rochester, New York clambakes, attack rates for those eating baked clams was 18%; for raw clams 60%, and for raw and baked clams 80% (161).

This indicates that in actual field situations, temperatures used to steam or cook shellfish are often insufficient to inactivate NLVs. At the Rochester clambakes, the clams were prepared from raw clams on the half shell, topped with a breaded "casino mix", and allegedly baked at 177° C for 20 minutes. The casino mix consisted of bread crumbs, green peppers, pimientos and other seasonings, a mixture which may have insulated the oyster meat from the heat. In spite of this cooking time, some persons who ate the clams considered them undercooked (161). In a Louisiana outbreak, 33% of persons who ate an oyster stew became ill. Oysters for the stew were reportedly sautéed until "brown and shriveled" before being added to the stew. Of 11 persons who ate only fried oysters, eating 3-24 oysters each, none became ill (100).

McDonnell et al (116) investigated the effect of cooking in the large Appalachicola outbreak. They interviewed persons involved in the outbreak as to the subjective level of doneness of their cooked oysters. While temperature could not be ascertained, the levels of doneness were classified as less done, which included oysters described as "wet and slippery" and "moist and juicy" and more done, including oysters described as "firm and dry" and "tough and dried out". They report high attack rates in all persons who ate cooked oysters, with attack rates not differing significantly between less done (71%) and more done (53%). Kirkland et al (98) also found no relationship between attack rate and the extent to which oysters were cooked in another outbreak investigation. While this doneness measure is subjective, it suggests that the level of cooking that would be

required to inactivate NLVs might render the oysters unpalatable to consumers (3, 99, 116). Protection of the shellfish beds from fecal contamination offers the best defense against shellfish-borne NLV.

Virus particles have been shown to survive the standard cooking practices because adequate internal temperatures may not be achieved throughout the preparation of the shellfish dish. Viruses are known to be inactivated by heat, which causes the coagulation and breakdown of the virus protein coat, or capsid. However, the medium in which viruses are held has been shown to influence virus sensitivity to thermal inactivation (45). Using poliovirus model to test various cooking methods, DiGirolamo (45) demonstrated that 10% of poliovirus innoculum could be recovered from oysters after 8 minutes of stewing in milk, with a final internal temperature of 75° C; 8 minutes of frying oysters in vegetable oil to a internal temperature of 100° C left 13% virus surviving ; oysters baked for 20 minutes to an internal temperature of 90° C left 12.7% virus surviving; and 7-13% of poliovirus innoculum added to oysters survived 30 minutes of typical steaming with a final internal temperature of 94° C (45). Other studies reported that it took four to six minutes of steaming for the internal temperature of soft-shell clams to reach 100° C, but only about 60 seconds for their shells to open, which is the standard often used to determine whether shellfish are ready to serve (99, 124). Using feline calicivirus (FCV) as a model for NLV, Slomka and Appleton (149) showed that in experimentally contaminated cockles, immersed in boiling water for 30 seconds, and at a mean internal temperature of 62° C, FCV remained infective in tissue 4/4 cases, though the titer was reduced 100-fold. When cockles were boiled for 1 minute, to a mean internal temperature of approximately 78° C, FCV survived in 0/7 cases. Norwalk-like agents

have been shown to be relatively heat-stable and can survive at a temperature of 60° C for 30 minutes (48, 161). Neither poliovirus, which is more heat sensitive than NLV, nor FCV, appear to be accurate models for NLV heat inactivation studies (48, 149). More work is needed to define the time/temperature requirements for inactivating NLV in foods.

Consumption of other foods, such as beer, crackers, and hot sauce which has an acid pH, at the same meals with the contaminated shellfish has consistently been shown to have no protective effects (25, 49, 98, 116).

Dose-response relationships have been observed . Dowell et al (49) report an attack rate of 40% among those who ate 1-5 oysters, 68% among those who ate 6-17 oysters, and 77% among those who ate 18 or more, ($p=.16$ χ^2 for linear trend) among oyster eaters at a large festival. A dose-response was also observed in an outbreak where 45 became ill after eating raw oysters. The attack rate was highest among those who had consumed more that 5 dozen oysters (91%) and lowest among those who had consumed less that 12 oysters (46%), (χ^2 for trend=3.98; $p=0.05$) (25). Morse (124) presents a similar finding; attack rates were 34% among persons consuming 1-3 raw clams, 50% among those consuming 4-9 clams, and 59% among those consuming 10 or more (χ^2 for trend = 13.92, $p<0.001$).

Current standards for tagging and identification of oyster lots are not always diligently applied, and do not guarantee rapid tracing for the purposes of identifying and destroying contaminated shipments. Oysters can be traced to their harvest beds because of the regulation requiring sacks of oysters to carry a tag identifying the harvester's ID number, the harvest date and the general harvest areas from which they are taken, and the tags must be retained for 90 days after sale. However, they are not sufficiently detailed to

allow recall of oysters from a specific site, and they can be lost during shucking (26).

Oysters pass through a complex commercial network, from the harvest area, to distributors, to large packers and shippers, to wholesalers, retailers and consumers (49). Outbreak-related tracebacks have also revealed that records from wholesalers often do not agree with the information on the oyster sack tag, and that harvester ID numbers cannot be consistently traced to harvesters (23, 26, 49). Further, there is no mechanism for forward tracing of oyster shipments once the source of the contaminated oysters has been identified. Dowell et al (49) report that in the 1993 Louisiana outbreak inspectors were able to document which merchant had had implicated lots of oysters confiscated or destroyed, but could not account for most of the oysters which had been shipped. Most state shellfish programs do not routinely collect information about the number of oysters or boxes destroyed during a recall or about the number of potentially contaminated boxes received and distributed prior to notification about the recall (49).

Since shellfish can be shucked, frozen and sold at a later date, contaminated oysters may be available to consumers for months following recognized outbreaks. In a large outbreak of NLV gastroenteritis in Australia, two further clusters of illness occurred 6 months after the original outbreak was identified, and were associated with consumption of frozen oysters from the same lot (49). These field observations of the viability of frozen virus are supported by laboratory research. Much earlier studies using the poliovirus model demonstrated that 13% percent of virus innoculum still persisted after 30 days in refrigerated (5°C), but by that time decomposed, oysters. In oysters held frozen at -17.5°C, 10% of innoculum survived 12 weeks of storage (45). In another outbreak, a large batch of NLV-contaminated cookies was frozen, and then portions were

subsequently served at different times to groups of people, causing a series of related outbreak clusters over a period of fourteen weeks (120).

Theoretically, screening oysters for viral contamination might help protect eaters of raw oysters. Current techniques relying on fecal coliform testing are an insensitive indicator of viral contamination (25, 49, 100, 118); direct detection of NLVs would be preferable. Kohn (100) reported that in the Cabbage Reef outbreak, NLV were not detected in implicated lots of oysters by RT-PCR. Current RT-PCR techniques in oysters have a lower limit of detection of 50-500 NLV particles when oysters are seeded with known quantities of virus in the laboratory (6,100). This lower limit of detection appears to be much higher than the infectious dose of NLV, which is thought to be about 10-100 virus particles. Also, the detection of NLVs in oysters contaminated in the wild has not been reported. Natural substances occurring in the oyster homogenate which is tested may also interfere with the ability of RT-PCR to amplify the viral genome (100, 149).

In the absence of detection techniques, recommendationss to prevent further shellfish-borne outbreaks of NLV might include: improved surveillance and reporting of shellfish-related outbreaks of gastroenteritis; embargo shellfish implicated in disease outbreaks; adopt strict state and federal laws to control the sanitary quality of shellfish (the new seafood HACCP requirement is a step in this direction); increase participation in the Interstate Shellfish Sanitation Conference; provide an sufficient number of enforcement officers; develop adequate water quality standards which address viral as well as bacteriological parameters (using poliovirus or other cultivable, sewage-related virus, as an indicator); mandate a manifest-type tagging system; strictly enforce wholesale and retail tagging requirements; require depuration of shellfish sold (though this has yielded mixed

results in Britain); and increase educational outreach concerning the risk of consuming raw shellfish (35, 55, 72, 143, 163).

Infected Food Handlers

Although shellfish have been important regional vehicles for outbreaks of food-borne NLV-related illness, transmission from infected food handlers appears to be a very common and widespread phenomenon.

The dynamic nature of transmission by food handlers was demonstrated by an outbreak in a bakery in which a single food handler experienced the onset of symptoms on the way to work and had at least 5 episodes of diarrhea and 2 of vomiting throughout his 6 hour work day. During this time, he made 76 liters of butter cream frosting that was used on at least 10,000 frosted bakery items that were sold to the public. Observation of the frosting preparation procedure showed that the baker preparing the frosting often submerged his bare arm up to the elbow in the frosting as it was being prepared to break sugar lumps and scrape down the side of the vat. At least 3000 cases, arising at a corporate picnic, a wedding reception, a graduation party, and among the general public, were attributed to this outbreak (74, 104).

In the majority of reported outbreaks that probably resulted from transmission by infected food handlers, a food handler who was ill before or while handling the implicated food item was identified. In 20 reported outbreaks reviewed here that probably resulted from transmission by food handlers, a food handler who was ill before or while handling the implicated food item was identified (see Table 3). Tossed salads, fruit salads or fruit slices were implicated in 13 (65%). Cold foods, such as smoked trout, tuna and turkey

Table 3. Summary of Foodborne Outbreaks of NLV Gastroenteritis

Reference	Number meeting case definition	Attack Rate epidemiologically	Foods implicated NLV of foodhandler	Illness status
Brondum (16)	29/58	50%	Sandwiches Quiche squares	Acute illness
CDC (29)	99/835	12%	Crumb cake pie, cinnamon rolls, ice cream	Acute illness
Fleissner (51)	350/700 26/87	50% 30%	Baked beans, chicken potatoes, meatballs, fruit salad, water	Acute illness
Gordon (56)	155/336	46%	Shrimp newburg bisque	Asymptomatic
Griffin (68)	38/41 25/31 71/118	92.7% 80.6% 60%	Green salad	Acute illness
Herwaldt (75)	217/527	41%	Fresh fruit Stuffed eggs	(Not stated NS)
Iverson (81)	250/280	89%	Melon, horseradish sauce, vermicelli consomme'	Asymptomatic and probable post- symptomatic
Kilgore (97)	188/363	52%	Salad	Acute illness
Kuritsky (104)	129/248	52%	Cake, frosting	Acute illness
Lieb (111)	277/790	35%	Tossed salad, ranch dressing, oil & vinegar	Asymptomatic
Lo (112)	195/NS	NS	Turkey salad sandwich Tuna salad, salad items	Presymptomatic
Parashar (133)	85/234	36%	Sandwiches	Postsymptomatic and asymptomatic
Patterson (139)	67/263	25%	Ham, coronation chicken	Postsymptomatic
Reid (141)	23/31 7/19	85% 31%	Smoked trout soup, salads	Acute illness

salad, coleslaw, baked goods, sandwiches were implicated in 7 (35%).

In three of these outbreaks, chicken, potatoes, and shrimp entrees were implicated as vehicles of NLV infection. Food cooking or holding temperatures were not reported. It appears that while these foods were served "hot", they were not held at temperatures hot enough to inactivate the virus particles (51, 56,139).

In 3 outbreaks, asymptomatic food handlers prepared the implicated foods (51, 56, 103). In one of these, the food handler had a sick infant at home, and also had a significant rise in antibody titer to Norwalk virus, but did not experience symptoms (51). This episode exemplifies asymptomatic virus excretion.

In other studies, asymptomatic persons associated with outbreaks have also demonstrated significant rises in anti-NV titers (76, 103, 133). Heun et al (76) proposed that their epidemiological data suggest that asymptomatic persons do not efficiently transmit illness.

In a 1998 study using more sensitive laboratory methods of detection, Parashar et al (130) detected NLV in the stools of an asymptomatic food handler who did not exhibit a positive immune response to NLV. Further, in recent volunteer studies NV was detected in stools of both ill and well volunteers. Viral shedding was detected in over 50% of well volunteers, and it persisted up to 2 weeks (130).

Graham et al (157) conducted a volunteer study to measure serological responses and viral shedding using new assays. Their work indicated an infection rate higher than expected, with a high rate of asymptomatic infection and prolonged viral shedding. Of 28 sujects, 26 (82%) shed virus and developed an immune response following challenge. One subject shed virus but did not seroconvert. Infection without any clinical symptoms

were seen in 8 (29 %) subjects, and mild symptoms were reported in 5 (18%). Thus, a large percentage (47%) of NLV infections may occur without any or significant symptoms (57).

More problematic are four outbreaks in which food handlers became ill after preparing or serving implicated food items. In an outbreak associated with green salads served at a restaurant, the outbreak was initially recognized because of illness among patrons of two luncheon banquets. Transmission occurred over a period of 6 days. One of two workers who prepared lettuce for the tossed salads reported onset of illness the day after preparing lettuce for the index banquets. This employee had a diagnostic rise in antibody titer to NV (68). Two other outbreaks occurred among students and staff eating lunches prepared in their school cafeterias. One of these was associated with sandwiches which appear to have been contaminated by a cafeteria worker who placed the sandwiches on plates without wearing gloves. This worker had a diagnostic rise in NV antibody titer but did not become ill until 36 hours after serving the implicated sandwiches (74). The second school-associated outbreak was associated with consumption of hamburgers and french fries handled 1-2 days before onset of diarrhea in two food servers who did not wear gloves while serving. Further, their contact with the implicated food items was reportedly restricted to handling pre-wrapped hamburgers and taking french fries with a scoop from a warming tray and placing them in plastic containers (74). Finally, an outbreak of gastroenteritis in two hospitals due to a NLV was associated with a food handler who became ill the day after preparing the implicated chicken sandwiches. This food handler had two children at home who were ill at the time (74).

In these outbreaks the reported onsets of illness in food handlers overlapped the distribution of illness onsets among patrons. Possible explanations for these observations include inaccurate recall, or lying on the part of the food handler regarding the onset of symptoms, transmission of the virus during the incubation period, or another unrecognized source of viral contamination. From the standpoint of implementing public health control measures, these scenarios have very different implications. In any outbreak investigation, it is essential to keep an open mind to the various possible modes of contamination, so as to permit consideration of all possible means of prevention. Although food handlers may seek to avoid blame for an outbreak and there is a natural tendency for investigators to be suspicious of food handlers who deny being ill, biases should be avoided. It is possible that a food might be contaminated prior to its arrival at the restaurant; produce which is chopped or shredded by the distributor could be contaminated. Such contamination at the distribution level could be missed if a source within the establishment's kitchen is assumed at the expense of other possibilities (74).

Post-symptomatic contamination has been implicated in several outbreaks (74, 81, 130, 133, 139, 141, 169). In a 1982 outbreak stemming from a hotel kitchen, two salad preparers who admitted to being ill were shown to have contaminated foods for up to 48 hours from the time their symptoms subsided. This was consistent with the shedding pattern of Norwalk virus previously reported in a human volunteer study (159). In this study, NV was visualized by immune electron microscopy in 2 of 11 stool specimens obtained 72 hours after onset of illness. Note that the sensitivity of IEM detection methods is 10^6-10^7 particles per gram; whereas the infectious dose of NLV is thought to be 10-100 particles. In another outbreak, where deboned cooked chicken was implicated,

it was found that the person who deboned and prepared the chicken without wearing gloves had been ill with NLV-like illness two days prior, during which time she stayed home. When she was recovered and asymptomatic, she returned to work and prepared the chicken, over 48 hours from the cessation of her symptoms (139). Reid et al (141) investigated a hotel outbreak lasting 8 days and found that the main vehicle of infection was cold foods prepared by a food handler during and after a mild gastrointestinal illness. The foodhandler was excreting NLV particles, detected by IEM, 48 hours after symptoms had subsided (141). Most recently Parashar et al (133) investigated a gastroenteritis outbreak among employees of a manufacturing company and found as association between disease and eating sandwiches prepared by 6 food handlers, 1 of whom reported gastroenteritis which had subsided 4 days earlier. The sick food handler's stool specimen was obtained 6 days after the lunch, or a total of 10 days after recovery from illness. A positive immune response was determined by IEM for sera from the sick food handler. These reports are consistent with recent findings on postsymptomatic viral shedding in human volunteer studies (130).

Early volunteer studies indicated that shedding of NLV occurs in <50% of ill persons and does not persist beyond 100 hours after initial infection (159). Polymerase chain reaction (PCR) analysis and new sensitive ELISA techniques indicate that viral shedding is probably more prolonged than previously recognized. In a recent volunteer study, NV was detected in both ill and well volunteers, by IEM, ELISA, and/or RT-PCR (130). It was found more frequently in unformed than formed stools, was maximal within the first 72 hours after exposure, and was present up to 13 days after challenge. NV was undetectable after 100 hours after challenge. Kaplan-Meier life table analysis showed that

after the first challenge, ill subjects shed NV for a longer period than well subjects. Subjects were challenged twice, 6 months apart and a third time 12 or 18 months after the first challenge. The duration of shedding was similar among groups after the second and third challenges. Further, NV shedding occurs in >90% of ill volunteers, and in over 50% of well volunteers, in whom in persisted up to 2 weeks (130). Similarly, Graham et al (57) found that virus particles are shed by infected symptomatic and asymptomatic subjects for at least 7 days.

In several outbreaks, a policy of unpaid sick leave maintained by the food service management has directly contributed to outbreaks, caused by workers who were sick and felt "compelled" to work (74, 169). This "compulsion" can come in two forms: the employee may not be able to afford to take time off, when pay would be lost; or the management cannot afford to have an employee absent from work due to the tight labor market and the lack of enough trained staff to fill in for the sick worker.

Hedberg and Osterholm (74) summarize the profile of NLV gastroenteritis outbreaks which were reported in Minnesota from 1981-1983. Salad items were implicated in 6 of these outbreaks, and an ill food handler was identified in 5 of those 6 outbreaks (103). From 1984-1991, an additional 39 outbreaks were reported. Cold food items were implicated as the vehicles in each outbreak; salad items were implicated in 12 outbreaks (35%). Ill food handlers were identified in 23 outbreaks (59%). In 6 outbreaks,(15%), the food handler who prepared the implicated food item was not ill but had ill household members. Thus, in 74% of outbreaks of viral foodborne gastroenteritis in Minnesota, a food handler source was identified. In 3 outbreaks (8%), food handlers had onset of illness at the same time as patrons, and no ill food handlers or other sources

were identified in 7 outbreaks (18%). These cumulative results of food-borne disease surveillance in Minnesota provide some perspective for evaluating the relative importance of transmission routes in outbreaks of food-borne viral gastroenteritis.

Control of outbreaks of food borne illness arising from food handler transmission requires removal of infected food handlers from contact with cold, lightly cooked, or ready-to-eat foods and food preparation surfaces, cleaning and sanitizing of contaminated surfaces and equipment, and disposal of contaminated food items. In several of outbreaks, multiple ill food handlers were identified, some being violently and repeatedly ill in the workplace. Of 18 restaurant-based outbreaks of food-borne gastroenteritis in Minnesota from 1984-1991, multiple ill food handlers were identified in 14 outbreaks (78%) (74). The frequency of transmission between food handlers within a kitchen, transmission from asymptomatic persons, transmission by persons who have recovered from illness, and apparent transmission from persons who are incubating infections make control efforts more difficult. The absence of paid sick leave benefits for most food handlers makes it economically disadvantageous for them to remain home when they are ill. Removal of cold food items from the menu controlled one outbreak; however, hot food items have been implicated in other outbreaks, and the potential for transmission from infected servers has not been thoroughly evaluated (74). The State of Minnesota recommends that ill food handlers be excluded from the workplace for 72 hours (74). However, excluding food handlers from work for 48-72 hours after recovery from illness may not always prevent transmission of virus, since virus may be shed for up to 10 days after the resolution of symptoms (133). Exclusion may not always be feasible. The 1999 FDA Model Food Code, which is being adopted by many states presently, requires

workers to wear gloves when handling ready-to-eat foods. While gloves can also be abused and contaminated, this offers a feasible and generally acceptable barrier to viral contamination, especially for unrecognized, asymptomatic cases.

When there is evidence of transmission of illness among the food handlers and transmission to patrons over several days, it may be advisable to recommend voluntary closure of the establishment for 72 hours to allow the virus to "run its course", and allow time for cleaning and sanitizing the equipment and facilities within the restaurant, and for disposing of potentially contaminated food (74). In Minnesota , 6 of the 18 (33%) of restaurants involved in outbreaks closed voluntarily due to the risk of ongoing disease transmission to patrons (74).

Waterborne Transmission

Data reviewed by Kaplan et al (90) suggests that NLVs may responsible for 23% of the waterborne outbreaks of acute gastroenteritis in the United States. The definition of waterborne disease outbreaks used by the CDC for surveillance purposes is restricted to illness that occurs after consumption or use of water intended for drinking (74). Classic outbreaks of waterborne viral gastroenteritis have typically been associated with private wells, small water systems, and community water systems. Several outbreaks arising from cross connections, or physical links between potable water systems and non-potable or waste water sources, have been reported (see Table 4). Taylor et al (157) describes an explosive outbreak of NLV gastroenteritis occurring at a school where 170 children were absent on the first day of the outbreak and 20-30 more were sent home sick. The investigation focused on the water supply when sports teams from other areas who had

Table 4. Summary of Waterborne Outbreaks of NLV Gastroenteritis

Reference	Site of Outbreak	Water Source	Details
Adler (10)	Public school	School well (presumptive)	Coliform count negative No cross connections found
Brugha (17)	Large bakery	Municipal water system	Cross-connection between municipal supply and river water found in nearby building. High coliform levels. Multiple strains of NLV detected.
CDC (29)	Campground	Well	Hydrant very near to septic tank. Dye test indicated septic leakage. High coliform count, no chlorine.
	Rustic lodge	Stream	Intermittent iodization of water, Filter removed from purification system. Stream water turbid. High coliform count.
CDC (30)	Commercial ice	Well	Well had been flooded by creek following torrential rains. Over 1000 cases of NLV illness.
Kaplan (92)	Textile plant	Municipal water	Cross-connection with industrial (surface water) supply. Over 1500 cases community-wide
Kukkula (102)	Community water	Lake water	Sand filtration with substandard levels of chlorine. High coliform count. Water contaminated by sewage outfall several miles away. 1500- 3000 cases of NLV illness.
Morens (123)	Resort camp	Spring water	Low chlorine levels. Spring was Contaminated by nearby septic tank; confirmed by dye test. 418 cases of NLV illness.
Taylor (157)	Elementary school	Well	Cross-connection between well and septic tank.

competed at the school, also became ill. It was highly probable that back-siphonage through a cross connection was the cause. Well water was pumped through a pressure tank through a ball-check release valve, with the desired pressure automatically maintained by on-off cycling of the pump. As the pump shuts off and the water column in the well shaft falls, a port in the valve opens to allow air into the system. As the pump turns on, and the water column rises, the air is expelled through the valve. As the valve closes, some water is spilled. Because spills were frequent, custodians had placed a plastic hose over the port and had run this to a floor drain. The floor drain had backed up near the time of the outbreak, resulting in 6" of sewage backflow pooling on the floor and submerging the end of the overflow hose. This resulted in the hose becoming a siphon when the pump cycled on and off, and sewage containing NLVs was introduced into the potable water system (157).

Contamination of potable water supplies by cross-connections with non-potable supplies have occurred so often as to necessitate the promulgation of state and local ordinances prohibiting cross-connections. Unfortunately, these ordinances often are not vigorously enforced. In many cases, financial stresses have reduced or eliminated cross-connection inspection activities among regulatory agencies, usually local building departments. In addition, cross-connections are often rigged after the final construction building inspection occurred, indicating that such inspections should be conducted on an ongoing basis to be most effective.

Groundwater contamination of wells has occurred as a result of municipal lagoon leakage, leakage from septic tanks, and flooding after heavy rainfalls. Well construction plays a role in the protection of groundwater; a properly constructed well with a pitless

adapter and grouted casing offers less possibility of allowing leakage to traverse the vertical well shaft to reach the aquifer. Older, unprotected construction acts as a conduit for surface pollution to reach groundwater, since water from the surface can flow down unto the earth unobstructed along the well pipe. The aquifer and overburden are also important factors in groundwater source contamination. A solid bedrock aquifer is best protected: clay overburdens are dense and add protection, while sandy soil or fractured bedrock are quite permeable. Wells should also be isolated from known potential sources of sewage, such as septic systems, both by distance and by higher elevation. Wells which are not properly abandoned are a real danger to aquifer protection. Properly abandoning a well involves removing the pump and backfilling the shaft with concrete or bentonite. Without this, the open well shaft is an unimpeded pipeline for pollutants to enter the aquifer from the surface. Trends toward increasing land application of sewage sludge over land which may contain abandoned farm wells could create major sewage contamination of groundwater.

While groundwater is generally regarded as the purest and safest source of water, sewage contamination of groundwater has resulted in outbreaks of NLV-related illness (18, 29, 30, 74, 105, 114, 123). An outbreak of NLV-like illness in Michigan in1970 led to the first recovery of virus particles from drinking water. Mack et al (114) concentrated vaccine strain poliovirus by using an ultracentrifuge, proving that sewage contamination of the well had occured. A large and interesting outbreak involving over 100 people occurred at a large Arizona desert resort, which had a state-of-the-art, five branched seepage sewage treatment system. When two of the leach fields became incapable of accepting effluent, the others were overburdened, and the leach fields became saturated.

The undertreated sewage water then percolated down through silt and sandstone bedrock into the water-filled caverns which were the source of the resort's potable water. The previously pure water in the deep bedrock caverns became contaminated and constant pumping was not sufficient to remove the contamination. The previously pristine groundwater could only be used for irrigation after this incident (105).

A sewage-contaminated well was also the source of a large NLV outbreak associated with ice. An outbreak involving hundreds of people in four outbreak groups including a university football game and museum fundraiser, was traced to a large commercial producer of ice (18). Prior to the outbreak, the manufacturer's water wells and septic tank were flooded by water from an adjacent creek after a torrential rainfall. Ice production was halted for 2 days and resumed after turbid water was pumped out of the wells. The wells were not chlorinated before production was resumed. The ice manufactured during this time period was associated with the massive outbreak. Several families who obtained their water from private wells along the creek also reported diarrheal illness that occurred a week after the flooding (18). A relatively high attack rate was present among those who ingested ice with alcoholic or carbonated beverages. Neither the alcohol content of alcoholic beverages, the acid pH of carbonated beverages, nor freezing affected the infectivity of the NLV (18). Indeed, freezing appears to have a preservative effect on enteric viruses in water (42, 102).

The identification of commercially manufactured ice as the source of this outbreak highlights the need for regulation of ice and bottled water production. In the investigation reported by Cannon et al (18), jurisdiction over recalling potentially contaminated ice and for ensuring the quality of ice production was unclear. Many containers did not carry

labels identifying the manufacturer or production date. Records of production and distribution should be maintained for traceback purposes (18). No overriding federal laws exist, and states vary widely in the regulation of the wells and the manufacture of ice and bottled water (Richard Overmeyer, Michigan Department of Environmental Quality, personal communication).

Surface water sources of drinking water, including springs and streams, have also been contaminated by runoff associated with heavy rainfall or by sewage pollution. Contributing factors in these outbreaks may have included the absence of filtration and the absence of or inadequate chlorination of the water supply (18, 29, 30). In Finland, outbreaks were caused by consumption of sand-filtered, chlorinated municipal water drawn from a frozen lake in which pollution incidents appeared to happen months apart, and over 70 km away (102). Research has shown that enteric viruses persist for many days in frozen-over surface water, often surviving longer than the indicator fecal strep and coliform bacteria which are commonly viewed as indicators of fecal pollution (42).

Studies have shown that NLVs are quite resistant to disinfectant in concentrations commonly found in drinking water, and also in experimentally elevated concentrations (95). In a volunteer study, dilute doses of NLV in distilled water were treated for 30 minutes with 3.75-6.25 mg/L sodium hypochlorite, yielding a free chlorine residual of 0.5-1.0 mg/L, as might be found in a drinking water distribution system; with 10 mg/L sodium hypochlorite solution, which approximates the chlorine level present when super-chlorinating a contaminated system; and with 0 mg/L. After the 30 minute treatment time the chlorine was neutralized with sodium thiosulfate. The group ingesting the samples with 0 chlorine had a 69% attack rate, those ingesting 3.75-6.25 mg/L had a 63% attack

rate, and those ingesting the 10 mg/L solutions had a 12.5% attack rate. The resistance of NLV to chlorination may be enhanced by the natural tendency of the virus to aggregate into clumps containing several viruses. Keswick reported that an outbreak of NLV-like illness was associated with stored water with a residual 0.7-1.0 mg/L of iodine (95). Thus, the routine disinfection treatment of NLV-contaminated source water has been shown to be inadequate to inactivate NLV, consistent with the findings in the Finland outbreak described by Kukkula (102)..

The role of water in outbreaks of viral gastroenteritis is broader than the CDC's surveillance definition implies. For example, an outbreak involving 1500 cadets and staff at the US Air Force Academy was attributed to the consumption of chicken salad. No ill food handlers were identified. However, celery used in the chicken salad had been washed and soaked for an hour in water obtained from a hose that had been used previously to unclog floor drains after sewage had backed up in to the kitchen (168). It was hypothesized that virus particles were rinsed from the hose and taken up by the celery during the wash and soak. Although this outbreak was food-borne, it appears that water played the critical causal role.

A major class of outbreaks associated with water not intended for drinking is made up of outbreaks associated with recreational waters. Outbreaks of NLV gastroenteritis have been associated with swimming in lakes and swimming pools (8, 60, 94, 101, 131).

Swallowing water or immersing the head in contaminated recreational waters increases the risk of illness (8, 60, 94, 101). These outbreaks typically occur during the summer months in the United States. Infected individuals contaminating crowded swimming areas can produce apparent point source outbreaks among exposed groups.

Potential sources of sewage contamination must also be investigated. Because of potential for ongoing transmission, it may be necessary to close implicated swimming areas for a minimum of 72 hours to prevent additional transmission.

An epidemiologic lead in determining whether the source of an outbreak is foodborne or person-to-person, or is related to gross contamination of sewage is the recovery of two or more strains belonging to NLV genogroups from victims. Sewage systems concentrate wastes from large numbers of households within communities where different genotypes of NLV are circulating concurrently, but rarely within the same victims. Several outbreaks in which raw sewage contamination has been implicated have resulted in concurrent infection with two or more NLV genogroups. This was demonstrated among canoeists who ingested river water (60), in consumers of oysters from contaminated waters (3, 100, 155), in potable water supply where heavy fecal contamination was evidenced at a drinking fountain, and a community outbreak with multiple genotypes of NLV resulted (17). Improving laboratory methods, mainly RT-PCR techniques which can test for multiple strains at once, will assist in epidemiological investigations by permitting the detection of mixed genogroups infections in victims, thus enabling a finer focus of investigational activities.

Person-to-Person Transmission

Person-to-person transmission, including direct contact, aerosol, and fomite exposure, generally has been reported in outbreaks involving elder care settings, hospitals, and cruise or military ships (33). In a study of 51 NLV outbreaks occurring in 1999 in the US, the CDC found that 20% were due to person-to-person transmission. About 10%

of reported NLV outbreaks in the US occur in extended care facilities for the elderly (56, 91). In Britain between 1992-1995, NLV accounted for 55% of all hospital and residential facility outbreaks reported to the surveillance system. Of these, 97% occurred in geriatric wards. The mode of transmission reported was mainly person-to-person in 93% of outbreaks in residential facilities, and a combination of foodborne and person-to-person in and additional 2.6% (43).

A pathognomic symptom of NLV gastroenteritis is projectile vomiting. Vomitus, in which virus particles have been identified by electron microscopy and RT-PCR, represents a major source of infection (65, 97). Because 10^6 virus particles/ml must be present for detection by electron microscopy, and if we assume that patients vomit a bolus of at least 30 ml, then 3 X 10^7 virus particles, with an infectious dose of 10-100 particles, will potentially be distributed into the environment (19). This is compounded as the number of vomiting episodes and the number of patients in the space increases. The result is the aerosolization of virus particles causing both airborne and environmental contamination. Evidence for respiratory spread is lacking, since replication of NLVs in respiratory mucosal cells has not been documented. However, there is evidence of airborne transmission via infectious aerosols, which impinge upon nasal and esophageal surfaces and eventually are swallowed (19, 34, 65, 78, 97, 146).

Chadwick (34) describes a tour group traveling on an air conditioned coach, wherein a lady vomited repeatedly on the outward leg of the trip, which included a 3 day hotel stay. Two days later, 15 other members of the party, including the bus driver, developed confirmed NLV gastroenteritis. Over the next 6 days, 3 other members of the bus group, 8 hotel guests, 10 hotel staff, and 2 environmental health officers became ill;

these were ascribed to secondary spread. After interview and analysis, no food item was implicated. Investigators concluded that illness was caused by aerosolized vomitus, which might have been further distributed by the bus coach air conditioning recirculation system (34). The predominant mechanism is practically impossible to discern; whether the aerosol is breathed in and swallowed to cause illness, or if environmental surfaces are blanketed with droplets which are then carried hand-to-mouth; probably both mechanisms occur simultaneously.

Aerosol spread has been reported on cruise ships, in elder care facilities, military facilities, and other densely occupied environments. Ho et al (78) describes an outbreak of NLV gastroenteritis aboard a cruise ship. There was no identifiable relation to food or water consumption, but the risk of gastroenteritis among passengers who had shared toilet facilities was twice that of those who had a private bathroom, and the rate of illness was related to the number of passengers sharing a communal restroom (i.e. one or more toilets). Contaminated bathrooms are an important vehicle for person-to-person spread of NLV (33, 78, 148). In each cabin, index patients who had vomited in their cabins were more likely to have cabin-mates who subsequently became ill than were patients who had not vomited. Similar findings were reported in other shipboard outbreaks, one of which continued through five consecutive voyages (71, 96). Many shipboard outbreak investigations are inconclusive as to the mode of transmission, since the shipboard environment is such a crowded and interrelated system (32, 71, 96).

Sometimes pertinent facts relevant to person-to-person transmission may elude investigators. After the conclusion of a major investigation of an outbreak of NLV aboard a cruise ship (71, 75), it was revealed by housekeeping crew members to a ship's officer

(author) that some cabin stewards would wipe down and polish the bathroom fixtures and drinking glasses with the same towel, since clean, sanitary replacement drinking glasses were hard to get in a timely manner. This sort of incidental, hidden behavior could explain how NLV environmental contamination can propagate illness from voyage to voyage after all passengers and many crew have changed over (71, 75, 78).

Distinct winter-spring seasonal patterns of occurrence (43) and the lack of seasonality (91, 93) in institutional outbreaks have both been reported. Due to crowded conditions, the infection can spread rapidly throughout the facility. Person-to-person transmission may be identified by temporal clustering. In an investigation of an outbreak in an elder care facility, Kaplan et al (93) analyzed the data in various ways. By using the attack rates and the number of rooms in the nursing home with 2, 3, or 4 residents respectively, the numbers of rooms in which 0, 1, 2, 3, or 4 persons would be expected to be ill given a random distribution of illness were calculated using the binomial distribution, yielding an analysis by geographic distribution. The observed numbers of double occupancy rooms with 0, 1, or 2 persons ill were then compared with expected values; no significant differences were observed. When illness was examined as a function of exposure to a roommate who had been ill one or two days earlier, by using a calculation of person-susceptible days, the relative risk for becoming ill one or two days after a roommate became ill versus becoming ill at other times was 3.74 (99% CI= 1.76, 7.96). Among the staff, significant differences in risk of illness existed for employees who reported daily physical contact with residents, compared with those who did not (χ^2= 8.64, $p<.01$). Kaplan cautioned regarding potential difficulties regarding the calculation of relative risk. The inclusion of susceptible-person-days for individuals who are not

actually susceptible (due to genetic resistance or immunity), and the presence of asymptomatic cases could distort the number of susceptibles. However, susceptible person-days attributed to such individuals would probably occur randomly during the outbreak period, and such random misclassification should not affect the relative risk (93). Other studies have supported these findings (56).

Among the elderly, NLV illness can have harmful consequences. Elderly people in extended care facilities are known to be highly susceptible to outbreaks of NLV gastroenteritis, probably due to decreased immunity and factors such as incontinence and poor hygiene (38). Gastroenteritis has contributed to the deaths of elder care facility residents by exacerbating heart conditions or diffuse athersclerosis. Hospitalizations have occurred due to severe dehydration, and to injuries such as scalp lacerations, broken toes and falls as elders attempt to make their way to toilet facilities during the acute illness (43, 56, 91, 93). NLV can thus hasten the death of an elderly debilitated person.

Actual airborne spread in controlled environments has been reported. Sawyer et al (146) report an explosive outbreak originating in a hospital emergency room where it was shown that housekeeping staff who merely walked through the emergency area became ill (relative risk=3.8, p=.029), as did friends and relatives of emergency patients who were waiting in adjacent spaces. Recent reports of NLV illness investigations in Alaska implicated airborne spread in three persons with tertiary cases who were exposed to secondary cases who were vomiting (33).

Although airborne transmission has not been positively demonstrated in food service settings, this failure is probably due to technical difficulties in confirming that transmission by aerosol occurred, rather than to the frequency of its occurrence (74).

Table 5. Summary of NLV Outbreaks Reporting Secondary (2°) Spread

Reference	Site	Primary source of NLV inf..	2° cases = % 2° Total cs. Cases	Details
Adler (1)	School	Water supply	120/372 = 32.3%	Household contacts.
Baron (8)	Park	Swimming in lake	21/111 = 19%	Household contacts.
Gordon (56)	Retirement facility	Foodborne	45/155 = 29%	RR=6.5 for roommate of 1° case.
Griffin (68)	Restaurant	Foodhandler	12/38 = 31.2%	Household contacts of primary case.
Heun (76)	School	Foodborne	21/48 = 44%	RR=5.5 for household contacts of primary case.
Ho (78)	Cruise ship	Not specified	Total cases = 237	RR=2 for those using common toilet facilities vs. private toilet facilities. OR=14 if roommate vomited in cabin.
Kaplan (93)	Nursing home	Not specified	15/45 = 33.3%	RR=3.74 of 2° infection if roommate is 1° case.
Kappus (94)	Municipal pool	Fecal contam.	117/229 = 51%	OR=12.1 for household of index case to have 2° case.
Khan (96)	Cruise ship	Ice	48/183 = 25%	RR=1.6 for occupying a cabin previously assigned to 1° case.
Koopman (101)	Municipal pool	Fecal contam.	5/21 = 23.8%	Household contacts.
Morens (123)	Resort camp	Well water	33/96 = 34%	Household contacts.
Sawyer (146)	Hospital	Not specified	48/183 = 25%	RR=3.8 for walking thru contaminated area.
Sharp (148)	Aircraft carrier	Not specified	91/336 = 27%	Common toilet rooms, water use restrictions, crowded conditions.

Secondary spread among household contacts is considered a feature of NLV outbreaks: 8 out of 9 outbreaks reviewed by Kaplan et al (91), and several other outbreaks reviewed herein described secondary spread (see Table 5). It is of note that reporting secondary when secondary spread was not mentioned in the scope of the study; "not reported" does not necessarily mean "not present". In an investigation of an outbreak of NLV in a school which specifically examined household secondary spread, secondary cases were found to occur in 44% (21/48) of households in which there was at least one primary case (76). The risk of secondary illness was 5.5 times higher in households with sick school children or adults than in households with well school children or adults in that outbreak. The secondary illness rate increased as the number of primary cases in the household increased. The risk of secondary illness was related to age of those susceptible: the preschool secondary illness rate was more than double the adult secondary illness rate. This increased risk among younger children has been observed elsewhere (157). Neither the age of the primary case nor the number of persons in the household had any effect on secondary transmission (76).

The characteristic duration (5-9 days) of most outbreaks reviewed suggests that outbreaks of Norwalk gastroenteritis terminate naturally in about one week. In a review of several outbreaks in camps and cruise ships, continuing exposure to a common source was responsible for longer outbreaks or repeated waves of illness when new susceptible populations were introduced (91). Outbreaks of this nature have been interrupted by early recognition and correction of deficiencies (such as cross connections), suggesting that interruption of the longer outbreaks is possible. However, there is little evidence that the course of a typical week-long epidemic has been altered by preventive measures (91).

Dispersing the susceptible population has been seen to interrupt ongoing outbreaks of NLV illness (146,148). When shore leave was granted to sailors aboard an aircraft carrier which was in the midst of an explosive outbreak, crowding was reduced significantly and the number of new cases declined substantially. When the crew returned after two days, the number of new cases spiked again, and the outbreak resumed. After a second major shore leave at another port some 18 days later, the epidemic ceased (148). In a hospital-based outbreak, the closure of the emergency room, which was the epicenter of infection, may have facilitated control of the epidemic (146). While potentially effective, as when the residents of London fled from the cholera in John Snow's day, dispersing the susceptible population seldom represents a practical control measure (148).

Once the index case of NLV is identified in a residential facility, generally presumptively by sudden projectile vomiting, immediate isolation of the case and the area is essential to prevent spread. Enteric precautions and attention to deep environmental cleansing of hard surfaces with hypochlorite (including high risk areas such as toilets and toilet rooms) is essential to containment. Contaminated and potentially contaminated linens from adjacent beds, and other removable fabrics should be rapidly removed and laundered and exposed consumables such as fruit should be discarded. Anti-emetics, administered intra-muscularly, rapidly terminate the vomiting, lessen dehydration, and act as a sedative, reducing patient mobility. Nursing staff are at risk of aerosol exposure, and respiratory masks may be considered. Handwashing and the use of gloves must be fastidious. Staff working in a ward where NLV illness is present should not work in other wards. Because NLV outbreaks are so common, infection control policies should consider environmental contamination as a routine program (19, 22, 141). In the home setting,

environmental decontamination, rigorous attention to hand washing, and segregating the patient from other family members may curtail spread of illness.

Chapter 6

SURVEILLANCE

Foodborne diseases cause an estimated 76 million illnesses and 5000 deaths in the United States each year (33, 117). Although foodborne illnesses are common, only a fraction of these illnesses are routinely reported to the CDC because a complex chain of events must occur before a foodborne infection is reported; a break at any point in the chain will result in a case not being reported. In addition, most reported foodborne illnesses are sporadic in nature; only a small number are identified as being part of an outbreak and thus are reported through to CDC. Most outbreaks are never recognized, and those that are recognized often go unreported. The likelihood that an outbreak is brought to the attention of public health authorities depends on many factors, including consumer and physician awareness, interest, and motivation to report the incident. The thoroughness of the investigation is conducted is influenced by the financial and personnel resources available to the agency conducting the investigation, and by the consultative and surveillance support of other local, state and federal public health and environmental agencies. Outbreaks that are most likely to be brought to the attention of public health authorities include those that are large, interstate, or food-service related, or that cause serious illness, hospitalization, or even death (33, 117).

Front-line surveillance for illness caused by NLV's and the detection and investigation of outbreaks in the US often falls to local health jurisdictions. Local health departments may or may not collect fecal specimens during the course of the outbreak investigation, and may only arrive at a presumption of NLV causation after routine

laboratory analysis has eliminated bacterial pathogens as the source of the illness. Many areas in the US are not served by laboratories with the capacity for conducting PCR analysis for confirmation of the presence of NLVs; at this time, only about 17 State and Regional government laboratories perform these tests. (Steve Michalik, Michigan Department of Community Health, personal communication, 33). Thus, the reporting of NLV related illness is greatly hampered by the relatively mild symptoms which seldom require medical treatment, the lack of recognition of an outbreak event, lack of resources and/or expertise in local health departments to conduct thorough epidemiological investigation, and lack of access to necessary laboratory services.

Presently, data in the US is collected through several systems administered by CDC. The Public Health Laboratory Information System collects passive national surveillance data for a wide range of diseases reported by physicians and laboratories, and the Foodborne Disease Surveillance System receives data from all states on recognized foodborne illness outbreaks. The National Health Care Survey, including the National Hospital Discharge Survey, the National Ambulatory Medical Care Survey, and the National Hospital Ambulatory Medical Care Survey, measure health care use in various clinical settings, and collect information on patient characteristics, patient symptoms or reasons for visit, provider diagnosis, and other information. Up to three symptoms are recorded, and diagnoses are recorded using International Classifications of Diseases, 9th revision (ICD-9-CM). Norwalk virus is coded as 0008.63, and other small round structured viruses are coded as 008.64 under ICD-9-CM. Information on food related deaths is collected by the national Vital Statistics System (117). The Foodborne Diseases Active Surveillance Network (FoodNet), established in 1996, conducts active surveillance

for seven bacterial and two parasitic foodborne diseases within a defined population of 20.5 million Americans (170). Additional surveys conducted within the FoodNet catchment area provide information on the frequency of diarrhea in the general population, the proportion of ill persons seeking care, and the frequency of stool culturing by physicians and laboratories for selected foodborne pathogens. NLV gastroenteritis is not included in this surveillance. It has been proposed within CDC to establish a similar "CaliciNet" active surveillance network to more accurately assess the true burden of Caliciviral disease, including NLV gastroenteritis (22, S. Monroe, CDC, personal communication).

In a recent summary of foodborne diseases from 1993-1997, the Centers for Disease Control reported that of the 2751 reported outbreaks, only 9 were caused by laboratory-confirmed NLVs. The number of outbreaks which were confirmed generally increased by the year during that time period. It must noted that 68% of the total outbreaks reported were of unknown etiology, and approximately 50% of those had an incubation period of $\geq$ 15 hours (33).

Surveillance of waterborne diseases is similar to that of foodborne diseases, and suffers from the same shortcomings. Outbreaks which are recognized are reported to the CDC, and summaries of waterborne diseases are released periodically by the Centers for Disease Control. For the period of 1991-96, three summaries were published. During 1995-1996, one outbreak of NLV gastroenteritis was associated with drinking water wells, and one with contaminated recreational waters (173). During the same time period, 8 out of the 30 reported outbreaks (36.4%) were of unknown etiology. In the two previous reports, covering 1991-92 and 1993-94, no outbreaks were attributed to NLVs,

and 23/34 and 5/34 outbreaks respectively, were of unknown origin (171, 172). Improving methods of detection and laboratory funding may improve the detection of NLV-associated outbreaks, and hopefully reduce the number of outbreaks of unknown etiology.

Chapter 7

BURDEN OF DISEASE

Early on, researchers recognized that the burden of disease caused by NLV was likely to be greatly underestimated. Kaplan et al (90, 91) developed criteria based on epidemiological and clinical characteristics of NLV illness: stool cultures negative for bacterial pathogens, and mean/median duration of illness 12-60 hr, and vomiting in 50% of cases, and an incubation period of 24-48 hrs. Using these criteria to analyze CDC data, Kaplan et al estimated that up to 45% of non-bacterial gastroenteritis was caused by NLV, a figure which greatly exceeded with CDC's estimate of 4% (90, 91). Kuritsky et al (103) analyzed data collected within the State of Minnesota reported from January 1981 through December 1983 using Kaplan's criteria, finding that 35% of outbreaks were consistent with NLV etiology. With the advent of novel and sensitive laboratory methods, researchers were able to offer better estimates of the disease burden. Fankhauser et al (50) analyzed fecal and emesis specimens from 90 outbreaks of non-bacterial gastroenteritis of undetermined etiology reported to CDC between January 1996 and June 1997. NLVs were detected by RT-PCR in 86 (96%) of the 90 outbreaks. Using similar laboratory methods, Vinje et al (165) attributed 91% of nonbacterial gastroenteritis outbreaks in The Netherlands to NLVs.

According to the Centers for Disease Control, NLVs are probably a much more important cause of outbreaks that is currently recognized. Using information from Foodborne Diseases Active Surveillance Network, the national Notifiable Disease Surveillance System, the Public Health Laboratory Information System, and many other

surveillance networks, Mead et al (117) formulated estimates of incidence for important pathogens causing gastroenteritis, including NLVs. Known pathogens account for an estimated 38.6 million illnesses each year, with the large majority, 30.9 million (80%) attributed to NLV, rotaviruses, astroviruses, and HVA. Of the 30.9 million cases attributed to these viruses, 23 million cases (73.3%) are attributed to NLVs. Mead et al estimate that 46.1% of all gastroenteritis caused by common pathogens is foodborne; 40% of all NLV cases are estimated to be foodborne (33, 117).

Thus, NLVs are estimated to cause 66.6% of all foodborne illness. Illness caused by NLVs are estimated to cause 50,000 (32.9 5%) of hospitalizations, and 310 (6.9%) of deaths related to foodborne illness (117).

Mead et al (117) used the FoodNet population survey, and self reported, age-adjusted data from the Tecumseh and Cleveland population studies to estimate the frequency of acute gastroenteritis in the general population. They estimated that in the US, there are 0.79 episodes of acute gastroenteritis per person per year. Extrapolated to a population of 267.7 million persons, (the 1997 US resident population), this rate is equivalent to 211 million episodes of gastroenteritis each year in the US. Using Mead's estimates to go one step further, if 59.5% of all gastroenteritis is caused by NLV, there may be 125.5 million episodes due to NLV per year (117). If only one day of work is lost per episode, the economic impact is substantial.

A profile of the use of the medical care system, loss of productivity, and economic impact of an outbreak of NLV was described by Kukkula et al (102). Investigating a waterborne outbreak of NLV illness, they surveyed the entire population of a region, some 5000 people, regarding their health during the time period of the outbreak. From this

data, they estimated that 1700-3100 persons became ill. Because of the abrupt onset and short recovery time, only about 50 people (1.6%-3%) sought medical care during the outbreak. They examined records of absences from school and work, and found that about 800 working days were lost; that is, 25-47% of those who were ill. The total costs, including medical care, were estimated to be about US$300,000, for the outbreak caused by this rather harmless virus (102). This comes to an average cost of $375 per person-work day. Using this figure, multiplied by 125.5 million one-day episodes in the US, multiplied by the lower figure of 25% of victims who miss work, means the cost of NLV illness may exceed $25 billion per year.

Chapter 8

SIGNIFICANCE OF NLVs

The significance of NLV illness lies in its role as a common cause of gastroenteritis. Traveler's diarrhea is one of the most common complaints among US military personnel who participate in exercises conducted in foreign countries, and it can affect enough personnel to compromise military effectiveness, especially during times of military mobilization (15, 80). Infection is often acquired during off-duty time when servicemen eat in local restaurants and have the same potential contact with endemic pathogens as other travelers. The development of a Norwalk-specific ELISA made it possible to assess the role of NLV in troop morbidity during the Persian Gulf War in 1990. In a study of 883 Marines deployed to Saudi Arabia and Kuwait, Hyams et al (80) found that 65% of troops reported at least one episode of gastroenteritis while in the Persian Gulf area. The overall rate of NLV infection during a 5 month deployment was 6/100 personnel (99% CI 4-9%). There was no difference in the rate of NLV infection by age, race or ethnicity, rank, birth location, or whether troops had previously been deployed outside the US. NLV infections were found to be a significant and widespread cause of acute gastroenteritis among US troops during the Gulf War. While the specific sources of infection could not be determined, person-to-person spread was important because of the crowded living conditions, and rugged living conditions in the desert in which latrines and communal bathing facilities were used (80)

Several outbreaks aboard US Navy ships have been reported, involving from dozens to hundreds of sailors (36, 132, 148). Corwin et al (36) describe a large outbreak

of NLV involving 450 personnel aboard an aircraft carrier, where the mean duration of illness was 37 hours, ranging from 3-96 hrs. The attack rate was 44%, and loss of work was reported by 39% of the ill population. In another large outbreak, involving 585/4500 crew members (13%), 8% of these sought medical attention and missed 1 or more days of work (161). Extreme demands were placed on the medical department, and all supplies of intravenous fluids aboard ship were depleted by the end of the epidemic. Close living conditions, limitations in the use of water for hygienic purposes, and common toilet facilities were again thought to contribute to the outbreak. In both outbreaks, recurrences in later months occurred aboard the ships, suggesting possible shipboard persistence of NLV over time, despite periodic ship-wide disinfection efforts (36, 132, 148). Fleet readiness was impacted by each of these outbreaks.

Among civilians, travelers' diarrhea has the potential for wrecking a meticulously planned business or vacation trip. Annually, 35 million people travel from industrialized countries to a developing country. Among them, the incidence of travelers' diarrhea from all causes was 20-50% per 2 weeks' stay in 1979-1981 (154). The increasing travel has resulted in dramatic changes in the tourism industry and put pressure on the local infrastructure that might influence the epidemiology of diarrheal illness. For example, in Jamaica, the number of tourists increased from 0.4 to 1.2 million per year during the 1980-1996 period (154)..

In a study done in Jamaica by Steffen et al (154), over 30,000 tourists were interviewed at the airport, and 322 travelers were enrolled at hotels to participate in the study, which lasted 15 months. Those at the hotels who became ill with travelers' diarrhea (TD) were invited to submit stool specimens in return for medical care. While NLV was

not among pathogens tested for, the data regarding days lost, quality of life assessments, and costs is applicable to NLV illness, which must surely be endemic in Jamaica.

The mean duration of visitors' stay in Jamaica was 4-7 days. The overall attack rate for any diarrhea was 24%, but only 12% had "classic" TD, defined as ≥ 3 unformed stools in 24 ours and ≥ 1 accompanying symptoms. Teenagers and young adults were most at risk, with rates at least twice as high as those aged > 55 yrs. British visitors had a higher attack rates (24%) than visitors from Canada (15%), US (11%), Germany (7.5%), Italy (4%), Japan (3%), and Latin America (1%). The British susceptibility was not explained, but lack of previous exposure or "so far undetected dietetic differences" were suggested (154).

Steffen also included "quality of life" dimensions, such as the degree of incapacity to function (in terms of leisure activities, sexual activity, and general well being). Almost half the patients with "classic" TD were incapacitated, compared with 9% of those with "mild" TD. The mean time of incapacitation was 12-17 hours (154).

Pathogen detection was 31.4%, when tested for a variety of bacterial and parasitic pathogens, rotavirus and adenovirus. Seasonal variations were observed, with higher rates (26-30%) of TD reported between May and October, 15% in December, staying below 20% until March. Viruses and *Campylobacter* spp. predominated in winter. Based on US foodborne illness estimates and rates of seropositivity world-wide, one may surmise that a large proportion of the cases of unknown etiology may have been caused by NLV. Few sufferers needed professional attention, and only two needed hospital admission, but the average cost for medication and missed activities was estimated to be $116.50 per patient per sick day (154). This does not include the irretrievably lost vacation time,

worry and frustration which is entailed in illness abroad.

Chapter 9

RISK FACTORS

Worldwide, diarrhea is a common and debilitating disease either directly or indirectly contributing to morbidity and mortality, particularly among children. Repeated episodes of acute diarrhea and vomiting or persistent symptoms can not only decrease quality of life, but also contribute to dehydration and malnutrition, and have adverse developments on development and survival. Children under 5 years of age in developing countries experience the highest rates of illness and death due to diarrhea, with the majority of disease occurring in infants under 1 year of age (137). While illness caused by NLV is relatively mild, it may contribute to the adverse effects of superimposed infections.

Knowledge about the role of NLVs in pediatric gastroenteritis in developing countries has been limited by the lack of available, sensitive and specific diagnostic techniques, and has probably been underestimated. Several recent studies document a high, age-dependent seroprevalence in young children, suggesting early and repeated exposure (46, 59, 86, 135, 151). In an urban Brazilian shantytown, Parks et al (137) isolated 7 unique strains, from both GI and GII genogroups of NLV from a cohort of 186 children over a 16 month period. The high number of strains detected in a short time suggest that there were multiple foci of infection in the community. Also, they found that infection with one strain offered no immunological protection against infection with other strains.

NLV diarrhea is more common in individuals of lower socio-economic status. Risk factors for these groups that favor the occurrence of diarrhea include overcrowding,

lower hygiene standards, and lower maternal education level, all of which favor oral-fecal transmission. A higher number of beds in the home (an indirect measure of crowding), younger maternal age, and consumption of seafood are additional risk factors (131, 137).

O'Ryan et al (131) studied differences between populations in two Chilean cities, and found that certain risk factors were significant in one city and not in the other. Child care center attendance and consumption of seafood were significant risk factors in Punta Arenas, yet not in Santiago. Thus, the predominance of a source of infection appears to depend on the habits of the population. They also found that in individuals with lower socioeconomic (SE) level, hygiene-related and food preparation habits are probably most important, while among members of higher SE groups, acquisition of infection is probably related to externally contaminated foods or water. This hypothesis is sustained by observations that swimming, and seafood and vegetable consumption were more significantly associated with seropositivity in individuals of higher SE groups (131). Despite the provision of universally potable water to all neighborhoods in Santiago, NLV infections were common, demonstrating that this measure is not sufficient for preventing NLV infections. Knowledge of food safety and general hygiene must be improved in order to intervene in the transmission of NLV to children at a very early age. The early use of supplementary foods, such as baby formula, into the diets of infants, is also associated with diarrheal illness (131, 137).

With regard to risk factors among travelers , Steffen et al (154) found that the old British colonial advice to "boil it, cook it, peel it, or forget it" was generally ignored by vacationers. Approximately 80% consumed dairy products and tap water, and more that 55% are ice cream, hamburgers, and incompletely cooked chicken, lobster or shrimp.

Less than 3% reported to have avoided all potentially contaminated food and beverage items. People aged 36-55 were slightly more negligent with regard to contaminated foods compared to other age groups. Those with TD scored 11.2 (out of 24) in the risky food score compared with 10.9 in those who did not have TD ($P < .012$). Tourists staying at all inclusive hotels, wherein all meals are provided, had a higher probability of diarrhea (OR, 1.58; 95% CI 1.26,1.96; $P<.001$). Despite the colonial rules of dietary discipline, consumption of high-risk "potentially hazardous" food was a weak predictor for the incidence of all diarrhea, and was no predictor for classic TD (154). This is consistent with the cold and ready-to-eat types of foods which are frequently associated with outbreaks of NLV foodborne illness.

Immuno-compromised persons are not only at risk for primary NLV infection, but also chronic infection may be established and has been documented among AIDS patients. In one study, an AIDS patient was found to shed the same genotype of NLV for an 8-month period. This shedding has been observed in chronic infections by other gastroenteritis viruses (106). Chronic infection with NLV may contribute to the deterioration of the general health of immunocompromised patients.

While the effects of normal cooking temperatures on NLV have been explored, research has been conducted as to the effect of novel pasteurization methods such as irradiation, electron beam, and ultra-high pressure pasteurization, which appear promising in the reduction or elimination of bacterial and parasitic pathogens contaminating the food supply. Unfortunately, none of these novel methods has yet been shown to inactivate viruses (L. Jaykus, CDC, personal communication).

The American military establishment is very interested in the development of a

vaccine to prevent outbreaks of gastroenteritis which have been seen to affect the battle-readiness of troops (7, 15, 36, 80, 132, 148). To date, the immune status of NLV-infected individuals has not been well defined and constituents of protective immunity are not known. At least one vaccine is currently being developed for testing in volunteers (7).

Chapter 10

SUMMARY

Norwalk-like virus infection is the epidemiologic prototype for outbreaks of foodborne and waterborne gastroenteritis. Worldwide, NLVs appear to be major causes of foodborne and waterborne illness. Until very recently, surveillance of NLVs throughout much of the world has been hampered by the lack of sensitive and specific means of laboratory identification, and today, many areas still lack facilities, equipment and trained personnel to conduct even passive surveillance. Assessment of the overall significance of NLV to the epidemiology of foodborne and waterborne illness depends upon the availability of routine laboratory services to confirm the viral etiology.

Outbreaks of NLV gastroenteritis have been propagated by contamination of water supplies, raw foods, and ill food handlers. Controlling an outbreak depends on identifying and eliminating the source of contamination. The occurrence of person-to-person transmission and the transmission of NLV via aerosols make it necessary to evaluate the potential for transmission by food handlers and servers in every outbreak, regardless of the original source.

The profile of an outbreak of NLV illness at the beginning of an outbreak may initially be indistinguishable from *Staphylococcus aureus* or emetic *Bacillus cereus* foodborne illness; thus all must be considered in the early hours of the investigation. Kaplan's criteria (90) for the diagnosis of gastrointestinal illness likely to be associated with NLV can be used to evaluate the outbreak profile. Kaplan's criteria are:
A mean or median duration of illness of 12-60 hours; a mean or median incubation period

of 24-48 hours; vomiting in at least 50% of cases; and stools negative for bacterial pathogens. Hedberg and Osterholm (74) also noted that having more cases present with vomiting than fever may be used as a further epidemiological criterion for NLV outbreaks.

In order to confirm the presence of NLV in foodhandlers or other suspected index cases, it is recommended that fecal specimens be collected from them at the outset, and held under secured refrigeration during the investigation until bacterial pathogens have been ruled out. By collecting the specimens at the outset, the chances of detecting NLV are greater, since days can elapse during the time needed for the laboratory to test specimens for bacterial pathogens. In this span of time, viral numbers in foodhandler's stools could decline to become undetectable, and the opportunity to confirm the source of the outbreak is lost. During an investigation, it is also important to remember that not only potentially hazardous foods, but any food which has been handled with bare hands can transmit NLV. From the very beginning, it is essential to coordinate the collection, holding, transport, and submittal of all food and fecal specimens with the laboratory, to prevent mistakes which can cripple the investigation.

While NLV is not the among the most virulent causes of gastroenteritis worldwide, it is indeed a very common one. Its ubiquity, the lack of protective immunity, and the ease and speed with which it spread make it an illness which is likely to be prevalent for some time to come.

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INDEX